MONOCLONAL ANTIBODIES IN DIAGNOSTICS

MONITORING OF MONOCLONAL ANTIBODY CHARACTERISTICS DURING (LARGE SCALE) PRODUCTION, PURIFICATION AND APPLICATION IN DIAGNOSTIC SYSTEMS

René van Erp

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Voor mijn ouders

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CONTENTS

Abbreviations

A_x	absorbance at x nm
Ag	antigen
BCIP	5-bromo-4-chloro-3-indolyl-phosphatase
BGG	bovine gamma globulin
BSA	bovine serum albumin
CBB	Coomassie Brilliant Blue
CD	circular dichroism
DASP	double antibody solid phase
DEAE	diethyl aminoethane
DMEM	Dulbeco's modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FCS	foetal calf serum
GaM	goat anti-mouse
hCG	human chorionic gonadotrophin
HEPES	N-2-hydroxyethyl-piperazine-N'-ethanesulphonic acid
HPSEC	high performance size exclusion chromatography
HRP	horseradish peroxidase
IEF	isoelectric focusing
IEP	isoelectric point
(m)IgG	(mouse) immunoglobulin G
(I)U	(international) units
K_a	affinity constant
LDH	lactate dehydrogenase
LH	Luteinizing hormone
(M)Ab	(monoclonal) antibody
MW	molecular weight
n	antibody valence
NADH	nicotinamide adenine dinucleotide hydride
NBT	nitroblue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PMSF	phenylmethylsulphonyl fluoride
r	moles of antigen bound per moles of MAb
RaM	rabbit anti-mouse
RIA	radioimmunoassay
RT	room temperature
SD	standard deviation
SDS	sodium dodecylsulphate
SPIA	sol particle immunoassay
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet

CHAPTER 1

INTRODUCTION

Antibody molecules

Antibodies are produced in all vertebrates as part of the immune response to antigenic challenge by foreign substances (antigens). The presentation of antigens to complementary structures on the surface of B and T lymphocytes is an early step in the sequence of events leading to the activation of the immune system. The receptor molecule on the surface of B lymphocytes, responsible for antigen recognition, is a membrane immunoglobulin. Contact with antigen results in the expansion of B cell clones specific for that antigen and in their differentiation into plasma cells capable of producing and secreting antibodies. These antibodies (immunoglobulins) bind specifically to the antigen by recognizing one or more sites - or epitopes - on the surface and initiate processes that can inactivate and eliminate the antigen. The diversity of this response is impressive: any foreign (macro)molecule can, under appropriate conditions, elicit an immune response. (reviewed by Harlow and Lane, 1988; Paul, 1989).

Apart from the natural function in the immune response, antibodies have long been an important tool for researchers who utilized the antibody specificity to label, or identify particular components and to separate them from a mixture. Before the hybridoma technology was developed by Köhler and Milstein in 1975, antisera for research and other applications such as diagnostics were polyclonal antibodies. However, the heterogeneity of the immune system has complicated the use of these polyclonal reagents. Since immunization of an animal stimulates many different B-cells, an antiserum will contain a mixture of antibodies of varying specificities and affinities; as a result cross-reactivity of polyclonal antisera is a major problem. In addition, the quantity and quality varies from animal to animal and good antisera are usually available in limited amounts.

Monoclonal antibodies, on the other hand, are eternally lasting (at least in theory), homogeneous preparations of immunoglobulin molecules. They can be produced (in tissue culture) for extended periods of time. These tailor-made monoclonal antibody molecules are produced from a single clone of a B-cell from the spleen of an immunized animal. Once fused with a mouse tumorcell, the resulting hybridoma produces and secretes antibodies of a single specificity and affinity that can discriminate between very closely related molecules, cells, and organisms. Cross-reactivity still occurs but less than in polyclonals. Moreover, the hybridoma technology enables the generation of a whole series of monoclonal antibodies that react with a particular antigen and to select those antibodies that will be the best for a particular application. Individual monoclonal antibodies can be selected amongst others on the basis of the affinity, specifity or

isotype. It is evident that monoclonal antibodies have some major advantages over conventional polyclonal antisera and the hybridoma technique along standardized methods (MAb production) has become a routine technique (Goding, 1986; Harlow and Lane, 1988).

Recent advances in both hybridoma technology and molecular biology has led to a new generation of MAb products. To date, the novel MAb products include chimaeric human/mouse MAbs (Morrison et al., 1984; Klausner, 1987a), bispecific MAbs (Songsivilai and Lachmann, 1990), single chain and single domain antibodies (Ward et al., 1988; Williams, 1988; Bird and Walker, 1991) and catalytic MAbs (Shokat et al., 1989). This new generation of recombinant MAb proteins show great promise in therapeutic and *in vivo* diagnostic applications. They have the potency of replacing the conventional hybridoma derived MAbs in other fields such as *in vitro* diagnostics.

Monoclonal antibodies in diagnostics

The advantages and potentials of monoclonal antibodies with respect to homogeneity, specificity, reproducibility and production mean that they are valuable tools for diagnostic applications; i.e. assays for the detection and measurement of hormones, drugs and infectious disease markers.

Both Scott (1985) and Sevier (1985) have, therefore, expressed the belief that MAbs will soon replace polyclonal antisera as reagents for immunological assays. However, the time and effort required in finding the appropriate MAbs for a particular purpose as well as the assay(s) which already operate satisfactorily with sufficient supplies of conventional antiserum make polyclonals not yet obsolete (Knight, 1990). Nevertheless, the specific advantages of MAbs and the large quantities in which these antibodies can be produced, result in a rapid replacement of the conventional antisera in standard kits for immunoassays in both biomedical and nonbiomedical fields (Klausner, 1987b). While many more MAbs become commercially available, the import of these new reagents as *in vitro* diagnostic tools will no doubt continue to increase.

Diagnosis of Pregnancy

An example of a typical diagnostic application taking full advantage of MAb specificity, sensitivity and the ability to identify different epitopes are immunoassays for human chorionic gonadotrophin (pregnancy tests) (Scott, 1985; Sevier, 1985). Human chorionic gonadotrophin (hCG) is a glycoprotein hormone secreted by the trophoblast cells of the placenta and is composed of two dissimilar α and β subunits with molecular

masses of 15 kDa and 23 kDa respectively (Bellisario et al., 1971, Carlsen et al., 1973). hCG has traditionally been used as a marker for the early detection and monitoring of pregnancy (Frances and Batzer, 1980). It is also used as marker for certain tumors of trophoblastic and non-trophoblastic origins (Halflants et al., 1990).

The incorporation of MAbs in the immunoassays for hCG has virtually eliminated the long time problem of cross-reactivity with luteinizing hormone (LH) (Sevier, 1985). In view of the high degree of structural homology between hCG and LH, it used to be difficult to obtain (conventional) antisera with high enough affinity and specificity for hCG. The commonly employed immunoassays for hCG involve agglutination assays (Leuvering et al., 1983; Halflants et al., 1990), radioimmunoassays (Sevier, 1985) and immunoassays using enzymes (Gupla et al., 1985; Tijssen, 1985) or fluorescent labels (Khosravi and Diamandis, 1987).

In this thesis, all investigations were performed using mouse MAbs directed against hCG.

1.2 Scope of the study

MAbs are often applied in diagnostics to obtain more sensitive, specific and reproducible test systems. Despite of the many advantages and large potentials of the Mabs over polyclonals with regard to these requirements, it has, however, become apparent that the MAbs are not eternally consistent and that reproducibility is not always ensured.

As the industry gains experience with the *in vitro* (large scale) production of proteins (MAbs), it is increasingly apparent that different cell culture conditions may lead to molecular alterations (Cotton et al., 1973; Bruggeman et al., 1982; Frame and Hu, 1989; Knight, 1990). It is recognized that proteins (MAbs) can be modified intracellularly by glycosylation and proteolytic reactions as well as extracellularly by processes that involve proteolysis, deglycosylations and deamidations (Manning et al., 1989; Moellering et al., 1990). Furthermore, it has been reported that MAbs are sensitive to changes in physical conditions such as pH, temperature and ionic strength (Underwood and Bean, 1985; Jiskoot et al.,1990). These effects may be manifest both in antigen-antibody affinity (Mosmann et al., 1980; Kranz et al., 1982; McCue et al., 1988) and irreversible structural (conformational) changes of the IgG molecule (Conradie et al., 1983; McCue et al., 1988; Jiskoot et al., 1990). Since the variety of purification methods encompass a wide range of chemical conditions, there are indications that adverse reactions to purified IgG were partially due to the particular method(s) of purification (Pirofsky, 1986; McCue, 1986 and 1988). Finally, immobilization of MAbs onto a solid support, as usually

employed in immunoassays, may significantly alter the MAb properties (Tijssen, 1985; Butler et al., 1986; Dierks et al., 1986; Lin et al., 1989).

In conclusion, the nature (structural integrity) of the final MAb product may differ from its naturally occurring counterpart as a function of its production, intra- and extracellular modification, purification, storage (stability) and immobilization to a solid support.

In order to predict the behaviour of a MAb in an immunoassay and to control test development and production, it is important to characterize, as much as possible, the significance of the modifications in terms of physicochemical and immunochemical properties. Since the affinity of the antibody/antigen interaction is of major importance in every immunoassay (Steward and Lew, 1985) particular interest is paid to the occurrence of changes in MAb affinity during the investigations.

The aim of this thesis is therefore: (1) to develop an appropriate method to determine the affinity constants of native MAbs for high molecular weight antigens in both buffered solution and culture supernatant and, (2) to monitor the characteristics of MAbs (directed against hCG) during (large scale) production, purification and application in diagnostic test systems. The latter involves determining which parameters need to be monitored in which phase, to control adequately test development and production.

The relevant aspects regarding MAb characteristics, production, purification and affinity (determinations), will be discussed in more detail, in the next paragraphs.

1.3 Structure of antibodies

Antibody molecules of the immunoglobulin G (IgG) class, the most abundant in normal serum, are composed of two identical light (L) and two identical heavy (H) polypeptide chains covalently linked by disulphide bonds to form a Y shaped structure. The amino terminal domains of the light and heavy chains are both responsible for antigen recognition. The antigen binding site of antibodies is formed almost entirely by six polypeptide segments, three in the variable domain of the light chain, and three in that of the heavy chain. These segments display variability in sequence as well as in number of residues, and it is this variability that provides the basis of the diversity in the binding characteristics of the different antibodies. These six hypervariable segments are also referred to as the complementarity determining regions or CDRs. (for comprehensive reviews: Tijssen, 1985; Edmundson and Ely, 1986).

The similar secondary and tertiary structure of all IgGs can lead to the misconception that monoclonal antibodies are biochemically similar. Although the essential basic structure and molecular mass of antibodies of the same class are similar, the differing primary sequence in the variable and hypervariable regions will alter the particular monoclonal antibody characteristics, notably with respect to isoelectric point (IEP), degree of hydrophobicity and charge density (Rothman and Warren, 1988; Schmidt, 1989). In addition, the nature and quantity of the carbohydrate moiety will also affect these characteristics (Hoffman, 1977; Burton, 1985; Rothman et al., 1989). Each monoclonal antibody should therefore be regarded as a different protein with its own properties. Even amongst monoclonal antibodies of the same subclass, considerable heterogeneity can excist; in a study of murine derived monoclonal antibodies, isoelectric points were shown to vary from 4.9 to 8.3 (Carlsson et al., 1985). This heterogeneity with respect to function and physicochemical properties is important, not only for their purification or conjugation but also for the application in diagnostic test systems.

1.4 Large scale production of monoclonal antibodies

Following the important development of the hybridoma technology, utilization of monoclonal antibodies in both diagnostic and therapeutic fields has grown dramatically (Knight, 1990). With this rising demand for monoclonal antibodies, the need for production systems and facilities to manufacture monoclonal antibodies on a large scale has increased. From a practical and an ethical point of view and for reasons of good manufacturing practice, monoclonal antibodies are preferably produced by *in vitro* cultivation. (Food and Drug Administration, 1987).

Until recently, the major limitation of traditional *in vitro* cell culture such as roller bottles and stirred bioreactors (Fazekas de St. Groth, 1983; Glacken et al., 1983) was low cell densities and, therefore, low product yields. In recent years there has been a major commitment to the development of new mammalian cell culture methodologies to overcome these problems. As a result, several novel cell culture techniques have been developed. These include among others the use of airlift bioreactors (Birch et al., 1985; Backer et al., 1988), hollow fibre technology (Hopkinson, 1985; Altshuler et al., 1986), ceramic matrices (Lydersen et al., 1985; Putnam, 1987) and micro encapsulation (Posillico et al., 1987). The monoclonal antibodies are secreted by the hybridomas into the cell culture supernatant which is recovered and processed.

The culture medium, however, is often supplemented with serum to stimulate cell growth and to increase monoclonal antibody production levels. The addition of supplements introduces contaminants which must be removed during the purification process. In order to eliminate this source of variability, efforts have been made to

develop serum and protein-free culture media (Murakami et al., 1982; Cleveland et al., 1983; Glassy et al., 1988; Butler and Jenkins, 1989). Consequently, utilization of these media wherever possible will significantly reduce the risk of contamination and lead to simpler purification processes.

The anti-hCG MAbs under investigation in this study have been produced using hollow fibre bioreactors as described by Schönherr et al. (1987). In this system hybridomas were grown to high cell densities ($>10^8$ cells/ml) in the extra-capillary compartment of dialysis modules with a constant supply of serum- and protein-free medium through the hollow fibres. The MAb production could be maintained for several weeks (up to three months) and MAbs were harvested in a concentrated form (1-10 mg/ml), free from serum components.

1.5 Purification

The increasing demands for more specific, sensitive and reproducible diagnostic test systems require (highly) purified IgG of a constant composition and free of contaminating proteins. These contaminants are a potential source of risk for non-specific reactions, stability problems and irreproducible results. Therefore it is important to make sure that the purity and quality of the MAb product are in the acceptable range.

As shown by the abundance of literature, a variety of methods are available for purification of MAbs. These include amongst others: ion exchange chromatography (Östlund et al., 1987; Posillico et al., 1987; Malm, 1987; Burchiel et al., 1984), Protein A (Goding, 1986; Lee, 1987) and Protein G (Ohlson et al., 1988) affinity chromatography; hydroxyl apatite (Stanker et al., 1985) and hydrophobic interaction chromatography (Danielsson et al., 1988), gel filtration (Philips et al., 1984) and salt precipitations (Tijssen, 1985; Reik et al., 1987). However, none of these methods can truly be applied generally, because of the heterogeneity in isoelectric point, hydrophobicity and size of the different MAbs. On the other hand it is obvious that all those techniques encompass a wide range of physical conditions that may affect particular MAb properties. (McCue et al., 1988, Jiskoot et al., 1990; see also Chapter 7).

As a result, purification methods are constantly being scrutinized and optimized to achieve more homogeneous MAb preparations. Up to now, most of the approaches to immunoglobulin purifications are, however, simple variations of the established purification methods. In addition, the most commonly used techniques for the isolation of murine monoclonal IgG from *in vitro* culture supernatants are protein A affinity chromatography, ion exchange chromatography and salt precipitation followed by gel filtration. These techniques are used as either a single purification step or a successive two step combination of these methods (Tijssen, 1985; Goding et al., 1986; Duffy et al.,

1989; Jiskoot et al., 1989, Perosa et al., 1990). Although a single purification step may yield sufficiently pure MAb solutions, a combination of two different purification techniques may be considered as a prerequisite to obtain highly purified MAb solutions.

1.6 Antibody-antigen interaction

The interaction of an antibody with an antigen forms the basis of all immunochemical techniques. An understanding of the physicochemical interaction and its mathematical description and the way it is influenced by external factors is of paramount significance for the quality of the immunoassay (Steward and Lew, 1985). The present section discusses the properties of the antigen-antibody interaction and is divided in the following three parts: affinity, calculations and methods to determine affinity constants.

Affinity

The binding of the antibody to the antigen is reversible and entirely dependent on non-covalent interactions. These non-covalent interactions include hydrogen bonds, van der Waals forces, coulombic interactions and hydrophobic bonds. The immune complex is stabilized by the combination of these interactions which depend on the precise alignment of antigen and antibody. The overall reaction is therefore a balance of many attractive and repulsive interactions at the interface; changes in either antigen or antibody binding site can affect profoundly the strength of the Ab-Ag interaction. (for reviews see: Burton, 1985; Getzoff et al., 1988)

The term antibody affinity is defined as the strength of the interaction between a single Ab binding site and the antigen determinant (epitope) and is quantitatively expressed by the affinity constant. The affinity for its antigen is one of the more important properties determining the usefulness of an (monoclonal) antibody. In general, high affinity antibodies are preferred in immunoassays such as ELISA, RIA, particle agglutination assays (reviewed by Steward and Lew, 1985), whereas lower affinity antibodies may be more useful for other applications such as affinity chromatographic purification of antigens where reversal of Ag-Ab binding is important (Morgan et al., 1984). Furthermore, a high afffinity antibody is superior to a low affinity antibody in a variety of biological reactions, for instance in the neutralization of infectious agents and in the elimination of antigens (Steward and Steensgaard, 1983; Steward and Lew, 1985).

On the other hand, a possible disadvantage linked to high affinity antibodies may be an increased level of potential cross-reactivity and a concomitant decrease in specificity for the homologous antigen (Karush, 1978).

The non-covalent and reversible binding reaction between an antigen with a single epitope and a single antibody binding site follows the basic thermodynamic principles of any reversible bimolecular interaction.

$$Ag + Ab \underset{kd}{\overset{ka}{\rightleftharpoons}} AgAb \qquad (1)$$

where Ab and Ag represent free antibody and free antigen respectively; AgAb the antigen/antibody complex; ka and kd the association and dissociation rate constants, respectively.

According to the Law of Mass Action;

$$ka/kd = K_a = [AgAb]/[Ab][Ag] \qquad (2)$$

where K_a is the equilibrium or affinity constant of the reaction.

Although it is also possible to define the equilibrium from the dissociation of the complex, i.e., $K_d = kd/ka$, it is more common to use the association equilibrium constant (K_a or K) as a measure of antibody affinity. This constant K_a has the dimension of l/mol and its value increases with increasing complex formation, a situation that will occur with high affinity antibodies. A low affinity antibody, on the otherhand, has a greater tendency for dissociation and the value of K_a, accordingly, will be lower.

Like all equilibrium reactions, the affinity constant for antibody-antigen interactions is affected by temperature, pH and solvent (ionic strength) (Tijssen, 1985). Changes in these parameters may change the affinity constant, either driving the reaction towards binding or dissociation.

Affinity is a thermodynamic expression of the primary interaction of a single epitope with a single antibody binding site which is independent of the number of binding sites. However, in the case of multivalent antigens and antibodies, the binding capacity of an antibody is usually expressed by the term avidity. Avidity is largely determined by affinity, but it also depends on Ab/Ag valence as well as on other properties such as Ab heterogeneity, net charge and hinge flexibility which are not directly concerned with the primary Ab-Ag interaction.

In this context, Karush (1978) suggested to avoid the term avidity and to use preferably the term functional affinity to describe reactions between multivalent antigens and antibodies. The important role played by valency in affinity measurements has been discussed in detail by Underwood (1988).

Calculations

The experimental basis for the measurement of the affinity of antibody-antigen reactions is the determination of antibody-bound antigen and free antigen at equilibrium over a range of antigen concentrations. A variety of mathematical equations have been developed to analyse the data obtained in such binding assays. The following equations are most commonly employed to determine affinity constants of MAbs (Tijssen, 1985; Steward, 1986; Connors, 1987; Devey and Steward, 1988).

The Langmuir equation:

$$1/r = 1/n \times 1/[Ag] \times 1/K_a + 1/n \qquad (3)$$

where r = moles antigen bound per mole of antibody; n = antibody valence; [Ag] = free antigen concentration at equilibrium; K_a = affinity constant. A plot of 1/r versus 1/Ag yields a straight line for homogeneous interactions and allows both n and K_a to be determined. The intercept on the y axis = 1/n and the slope, $1/nK_a$

The (modified) Scatchard equation (Scatchard, 1949; Feldman, 1972):

$$r/[Ag] = nK_a - rK_a \qquad (4)$$

A plot of r/Ag versus r yields a straight line for homogeneous binding and the intercept on the x-axis represents the antibody valence. The value of K_a can be obtained from the slope.

The Sips equation:

$$\log(r/(n-r)) = \alpha \log K_a + \alpha \log[Ag] \qquad (5)$$

where α = heterogeneity index. A plot of log (r/(n-r)) versus log[Ag] yields a straight line and the heterogeneity index (α) is given by the slope. The affinity constant is given by 1/[Ag] for log(r/(n-r)) = 0.

The mathematics of affinity constant calculations and the advantages/disadvantages of the different approaches have been discussed in detail by Steward (1986) and Connors (1987). As indicated by several authors (Connors, 1987; Ehle et al., 1989; Kermode, 1989), graphical determination methods have several features which lend them to convenient estimation of equilibrium (affinity) constants.

Alternative graphical representations of antibody binding data have been considered (Klotz, 1982; Singh et al., 1985; Connors, 1987) but none of the alternative plots, however, provides information on binding heterogeneity in as clear and succinct a manner as the (modified) Scatchard plot. Therefore, the (modified) Scatchard plot is the most frequently used method in calculating the affinity constant of MAbs (Scatchard, 1949; Ehrlich et al., 1982; Schots et al., 1988; Kim et al., 1989; Edmond-Rouan et al., 1989). The analytical drawbacks of the (modified) Scatchard plot should not be considered a reason for choosing a different graphical representation (Kermode, 1989).

Methods to determine affinity constants

A variety of methods have been described for the determination of antibody affinity constants. The experimental basis of many methods is the measurement of free and bound antigen (or antibody) concentrations at equilibrium. In general, these methods can be classified as involving either solution phase (both Ab/Ag interaction and bound/free separation occurs in solution) or solid phase assays. The solution phase assays include amongst others precipitation of complexes by adding various agents (Steward and Petty, 1972; Kim et al., 1975; Hetherington, 1988), separation of complexes based on size (Kim et al, 1989), changes in fluorescence properties of either antibody or antigen (Eisen, 1964; Karush and Tang, 1988; Friguet et al., 1989) and equilibrium dialysis (Giles et al., 1983; Klotz, 1989). However, these procedures are not generally applicable because some methods cannot be used with high molecular weight antigens whereas others are applicable only to purified antibodies. Furthermore, these described solution phase methods use labelled components which may considerably alter the molecules (Friguet et al., 1985; Radicella et al., 1985; Ramjeesingh et al., 1990).

The popularity of the solid phase assays, on the other hand, is due to the fact that a simple washing step replaces a variety of more cumbersome procedures for separating free from bound species. To date, most commonly employed solid phase methods to determine affinity constants are ELISA and RIA (Ehrlich et al., 1982; Nieto et al., 1984; Friguet et al., 1985; Beatty et al., 1987; Macdonald et al., 1988). Most of these methods, however, measure directly the interaction of the antibody with the immobilized antigen (or vice-versa) and therefore do not permit the measurement of the true affinity constant since antigen and antibody are in separate phases. Moreover, proteins tend to undergo

denaturation when adsorbed to a layer of plastic (Friguet et al , 1984, Dierks et al , 1986, Van Regenmortel, 1989) and steric hindrance at the solid phase may occur (Harper et al , 1987 Finally, successive incubation and washing steps may disturb the equilibrium (Stevens, 1987)

Considering all these methods it must be concluded that no appropriate procedure is available to determine the affinity constants of MAbs for large MW antigens both native and in aqueous solutions (buffer and culture supernatant). Therefore, a major part of this thesis deals both with the development of such a method and the understanding of the physicochemical aspects of the anti-hCG/hCG interaction.

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CHAPTER 2

APPLICATION OF A SOL PARTICLE IMMUNOASSAY TO THE DETERMINATION OF AFFINITY CONSTANTS OF MONOCLONAL ANTIBODIES

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Abstract

The affinity constants (K_a) of monoclonal antibodies (MAb) for binding to their corresponding antigens (Ag), unlabelled and in buffered solution were determined by the following procedure: 1. Incubation of MAb (fixed concentration) with Ag (concentration dilution series). 2. Rapid bound/free separation by adding immobilized second antibody, followed by centrifugation. 3. Determination of free Ag in the supernatant using a gold sol particle agglutination immunoassay (SPIA) in a microtitration plate format. 4. Calculations and interpretation were based on Scatchard and Sips plots. K_a values found by this procedure were found to be similar to those obtained by a radioimmunoassay (RIA) procedure. The present method avoids possible artefacts in K_a values introduced by the procedure or chemical modification due to labelling of MAb or Ag. It enables rapid, simultaneous screening of a considerable number of different MAbs under non-specialized (i.e. RIA) laboratory conditions.

Introduction

Monoclonal antibodies are widely used as primary tools for identification, assay and purification of a variety of antigens. The affinity of an antibody for its corresponding antigen is of crucial importance in the performance of immunoassays (Van Heyningen et al., 1982; Nimmo et al., 1984; Steward and Lew, 1985; Devey and Steward, 1988). The affinity of an antibody is determined at the equilibrium state and is quantified by an affinity constant. This affinity constant (K_a) is a measure of the strength of the interaction between antigen and antibody, and is a suitable parameter for the specificity of the antibody (Metzger et al., 1984).

The law of Mass Action provides the basis for the calculation of antibody affinity constants in a solution phase assay. A variety of mathematical equations have been developed to facilitate experimental calculations of affinity constants. The most commonly employed are the approaches described by Scatchard (1949) and Sips (1948). Utilizing these approaches, a number of experimental methods have been described for the measurement of the affinity constants of antigen-antibody interactions.

While equilibrium dialysis has become accepted as the reference method for the determination of affinity constants, it is not applicable to macromolecular antigens. Similarly, other techniques as radioimmuno- and solid-phase assays are less favourable since labelling or immobilization of the proteins may induce structural modification (Friguet et al., 1983; Radicella et al., 1985).

Friguet et al. (1985) introduced a method for the determination of affinity constants, that characterizes the interaction between non-labelled antibodies and macromolecular antigens. However, relatively large amounts of antigen are required and the affinity of binding sites on intact IgG is underestimated (Harper et al., 1987; Stevens et al., 1987).

The purpose of the present work was to develop a method for the determination of antibody affinity without applying labelled compounds in the primary (Ag-Ab) system. In order to study the interaction of native human chorionic gonadotrophin (hCG) and anti-hCG, the applicability of UV spectrophotometry, circular dichroïsm and high performance size exclusion chromatography (HPSEC) were examined. The first two techniques could not be used, however, to investigate hCG/anti-hCG interactions since no significant spectral changes occurred due to complex formation. The exclusion ranges of the commercially available columns and the low molar absorbance of non-labelled hCG at 206 nm were the limiting factors for HPSEC. Therefore, a technique was developed for the determination of antibody affinity (see Fig. 1) based on the SPIA agglutination procedure in combination with an existing bound/free separation technique (immunosorbent). The SPIA procedure was chosen above other immunoassays because of its reproducibility, low coefficient of variation (≤5%) and easy performance (one step) (Leuvering et al., 1980a). The K_a values obtained, were compared with a standard RIA procedure.

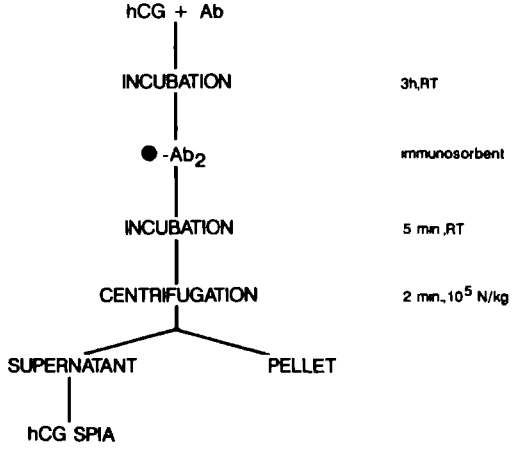

Fig. 1. Flow chart illustrating the principle of the method. In the first step a constant amount of MAb is incubated with various concentrations of Ag. In the second step a rapid separation of Ag bound and free is achieved by adding immobilized second antibody. After centrifugation the free Ag in the supernatant is determined using a gold sol particle agglutination assay.

Materials and Methods

Materials

The monoclonal antibodies used in this study were of the mouse IgG1 subclass and were directed against hCG, a glycoprotein with a molecular mass of 38 kDa (Bellisario et al., 1973; Carlsen et al., 1973). A detailed description of production of the monoclonal antibodies in hollow fibre dialysis modules has already been described (Schönherr et al., 1987). The MAbs were purified from hollow fibre culture supernatants by a 20% sodium sulphate precipitation (Tijssen, 1985) followed by a Sephacryl S-200 gel filtration (Pharmacia K26/100 column, eluent 0.15 M Tris/HCl, pH 7.6 + 0.2 g/l sodium azide). The isolated IgG fractions contained monomeric IgG (>98%) based on HPSEC (Zorbax GF-250 Dupont) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). HCG (12 100 IU/mg) was obtained from Diosynth Oss, The Netherlands. All other chemicals were of p.a. reagent grade quality.

Preparation of the Immunosorbent

The ("second") antibodies (rabbit anti-mouse) were coupled to regenerated cellulose by means of the reactive azo-dye procedure as described by Gribnau et al. (1979). The immunosorbent (DASP: 0.03 mg IgG/mg aminoaryl cellulose) was stored in 0.1 M Na_2HPO_4/KH_2PO_4, 20 mM NaCl, 5 mM EDTA, 1 g/l Thiomersal (pH 7.0) buffer at 4°C (dry weight: 24 mg/ml). Before use in binding analysis, the DASP suspension was washed four times with the assay buffer (0.05 M HEPES, 0.2 M NaCl, 0.1 g/l Thiomersal and 4 g/l BSA (Boseral-PUR, Organon Teknika B.V., The Netherlands) pH 7.3).

Determination of the Optimal DASP Concentration

The amount of DASP (rabbit anti-mouse (RaM) or goat anti-mouse (GaM) Fc specific) needed to separate free Ag from the free first MAb and the complex was determined according to the following procedure:

A constant amount of MAb anti-hCG (0.3 μg in 125 μl) was incubated in a total volume of 350 μl with different amounts of the DASP suspension. After incubation during 5 min and centrifugation (2 min; 100 000 N/kg), the total mIgG in the supernatant was determined using the SPIA agglutination procedure.

Sol Particle Immunoassay

The concentrations of hCG (i) and mouse IgG (ii) in samples of the supernatants were determined by the SPIA agglutination procedure as described elsewhere (Leuvering et al., 1980b and 1983). The quantitative SPIA uses gold sol particles coated with anti-hCG MAbs (i) or rabbit anti-mouse immunoglobulin (ii). The test was performed as follows:

100 µl sample or standard solution of hCG (i) or MAb (ii) and 50 µl buffered solutions ((i) 0.3 M Tris/HCl, 3 g/l BSA, 1.3 M NaCl, 0.1 g/l Thiomersal, 74 g/l PEG 8 000, pH 7.4 or (ii) 0.3 M Tris/HCl, 1.3 M NaCl, 0.1 g/l Thiomersal, 11 g/l PEG 8 000, pH 7.4) were pipetted into 96-well microtitration plates. After agitation, 50 µl buffered dispersions of (i) Au-(anti-hCG) or (ii) Au-(anti-mIgG) conjugate with A_{540nm} = 9.0 in 5 mM Tris/HCl, 0.05 g/l PEG 20M, 160 g/l sucrose, 0.1 g/l Thiomersal pH 8, was added and mixed by a plate shaker. After a 30 min incubation at RT, the extent of agglutination was determined by measuring the absorbance at 540 nm using a Titertek Twinreader. Wells filled with 200 µl Tris buffer were taken as blank.

Competitive Radioimmunoassay

Affinity constants (K_a) of the antibody-antigen interactions were determined in a sodium phosphate buffer solution (10 mM sodium phosphate, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 g/l bovine gamma globulin (BGG) Cohn fraction II (Sigma), pH 7.3) and by using labelled ^{125}I-hCG (Dupont NEX 106 specific activity 66.8 µC/µg). 500 µl of the antibody preparation (diluted to a concentration which bound 30% of 2 fmol ^{125}I-hCG added as determined in a titration experiment) was incubated with 100 µl of labelled hCG (10 000 cpm, 4 pM) and 100 µl of seven different amounts of unlabelled hCG (0 to 400 pM) for 16 h at RT. Separation of bound and free radioactivity was achieved using RaM DASP immunosorbent. Non-specific binding was assessed by competing the tracer with 1 000 fold excess of unlabelled hCG (Pregnyl 3 300 IU/mg, Organon, The Netherlands), without changing the total incubation volume. All experiments were performed in duplicate.

Determination of Ka by the Procedure using SPIA

MAb anti-hCG, at a constant concentration, was incubated with highly purified hCG at various concentrations (0-55 nM) in 0.25 ml of the assay buffer (0.05 M HEPES, 0.2 M NaCl, 0.1 g/l Thiomersal and 4 g/l BSA, pH 7.3). After incubation at RT (18-24°C) for 3 h

to reach equilibrium, an excess of the second antibody immunosorbent (DASP RaM) was added. The reaction mixtures were incubated for 5 min and continuously agitated. Subsequently, the reaction mixtures were centrifuged at 100 000 N/kg for 2 min using a Heraeus Biofuge centrifuge. The concentration of free Ag in each supernatant was determined in four fold by the SPIA agglutination procedure.

Protein Determinations

Protein concentrations were determined by absorbance at 280 nm using the following extinction coefficients: 1.45 $(cm \cdot mg/ml)^{-1}$ for IgG and 0.39 $(cm \cdot mg/ml)^{-1}$ for highly purified hCG. The validity of the values were confirmed by combination of absorbance measurements and amino acid analysis. The molecular masses of IgG and hCG were taken as 150 kDa and 38 kDa (Moyle et al., 1983) respectively.

Calculations

The "modified" Scatchard equation (Feldman, 1972) was used to determine affinity constants.

$$r/[Ag] = -rK_a + nK_a \qquad (1)$$

where r = moles of antigen bound per moles of antibody present; n = antibody valence (in this case 2); [Ag] = free antigen concentration; K_a = the affinity constant.

A plot of r/[Ag] versus r over a wide range of antigen concentrations yields a straight line for homogeneous binding and the intercept on the x-axis represents the antibody valence n. The value of K_a can be determined from the slope, which is equal to $-K_a$. If linearity is not observed over a defined range of antibody binding site saturation, the K_a value is not meaningful (Klotz, 1982; Connors, 1987).

Based on the Sips distribution function for the affinity constant, the following equation was derived (Karush, 1962)

$$\log(r/(n-r)) = \alpha\log[Ag] + \alpha\log K_a \qquad (2)$$

A plot of log(r/(n-r)) versus log[Ag] yields a straight line, and the index of heterogeneity, α, is given by the slope. The affinity constant (K_a) is given by 1/[Ag] when log(r/(n-r)) = 0. When the index $\alpha = 1$, there is no apparent heterogeneity. A value of $\alpha < 1$ corresponds to a Scatchard plot which is concave ("negative cooperativity") and $\alpha > 1$ is suggestive of "positive cooperativity" (Rodbard and Feldman, 1975; Byers, 1977). Since r is defined as moles of bound Ag per moles of Ab present, the result depends on accurate knowledge of the Ab concentration.

Results

The interaction between anti-hCG MAbs and hCG was studied by the procedure using SPIA. In this procedure Ab-Ag complexes in the equilibrated mixtures were separated from free Ag by using an excess of a second antibody immunosorbent (DASP). An adequate separation was achieved by the addition of 2.4 mg DASP as shown in Fig. 2.

The non-specific adsorption of Ag to the reaction vial wall and immunosorbent was suppressed by the amount of 4 g/l BSA in the HEPES buffer. Antigen binding was determined by measuring the concentration of free Ag after incubation of various

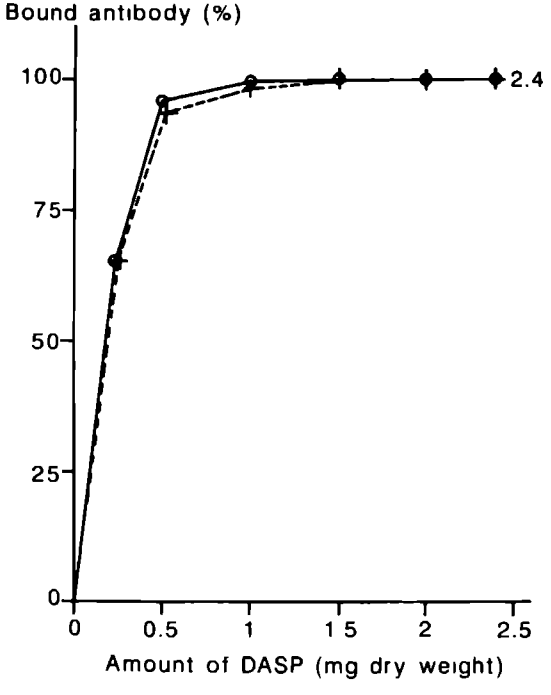

Fig. 2. The amount of DASP needed to achieve complete binding of MAb OT-7B at a concentration of 7 nM: (o) RaM and (+) GaM Fc specific. Similar results were obtained for other MAbs directed against hCG.

amounts of Ag with a constant concentration of Ab. The fraction of bound Ag was calculated by substracting the free Ag concentration from the total antigen concentration. It was found that incubation during 3 h at RT was sufficient to reach equilibrium as illustrated for MAb OT-0A in Fig. 3.

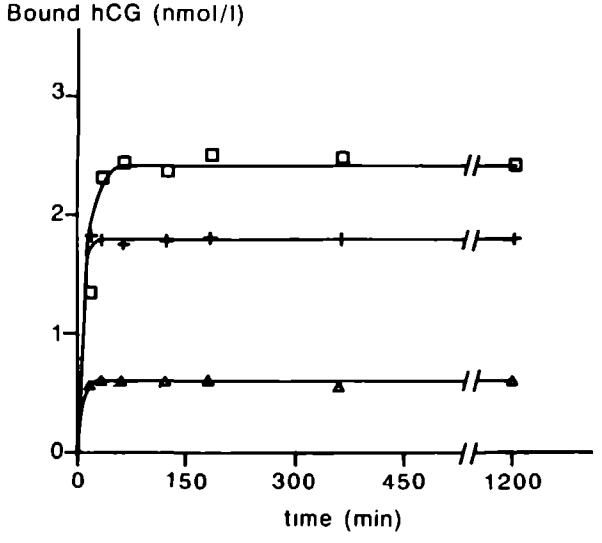

Fig. 3. The time for 1.3 nM MAb OT-0A to reach equilibrium binding at RT was measured with 0.72 nM (△), 2.16 nM (+) and 4.32 nM (□) of hCG.

Affinity constants for anti-hCG MAbs, determined by the procedure using SPIA, were compared with those obtained by a competitive radioimmunoassay. The results, based on Scatchard (1949) and Sips analysis (1948), were found to be similar and are summarized in Table I. The heterogeneity indices are not significantly different from one, which is in agreement with the proposed model of a homogeneous hCG/anti-hCG interaction.

In addition to linear Scatchard plots (characteristic for a homogeneous interaction) also convex Scatchard plots characteristic for cooperative interactions were obtained (see Fig. 4A). The Sips plots of these (hCG/anti-hCG) binding data, as shown in Fig. 4B, also indicated that an apparent positive (cooperative) site-site interaction was present, since the heterogeneity index was significantly larger than 1.

Table I
Comparison of affinity values obtained by the procedure using SPIA and a competitive RIA.

MAb[a]	Procedure using SPIA			Competitive RIA		
	Scatchard[b]	Sips[b]		Scatchard[b]	Sips[b]	
	$K_a \times 10^8$ (l/mol)	$K_a \times 10^8$ (l/mol)	Index (α)	$K_a \times 10^8$ (l/mol)	$K_a \times 10^8$ (l/mol)	Index (α)
OT-6A	0.1 ± 0.01	0.1 ± 0.01	0.98 ± 0.04	0.1[c]	0.1[c]	0.99[c]
OT-7B	19 ± 1	18 ± 1	0.98 ± 0.05	11 ± 2	12 ± 2	1.02 ± 0.03
OT-0A[c]	55	57	1.05	76	74	1.04
OT-3A-I	103 ± 7	100 ± 8	1.00 ± 0.04	90 ± 10	89 ± 9	1.05 ± 0.05

[a] The MAb concentration in the new procedure was about 1 nM and in the competitive RIA 0.2 nM. A concentration of 20 nM was used for MAb OT-6A.
[b] The results represent the mean value ± standard deviation of three experiments and are based on linear regression analysis.
[c] The values indicate the results of one experiment.

Contribution of Potential Artefacts

Deviation from linearity could be due to artefacts. This was ruled out by the following systematic investigations:

Starting materials. These observed convex Scatchard plots could not be due to aggregates in the starting materials since they were monomeric (>98%) as determined by HPSEC and SDS-PAGE.

Non-specific binding. Binding experiments, with both labelled and non-labelled hCG in the assay buffer containing 4 g/l BSA or 1 g/l BGG, showed that the non-specific binding was less than 4% of total binding. Since the convex binding characteristics were independent of correction for non-specific binding, it is very unlikely that the convex Scatchard plots were produced by low non-specific hCG binding. Moreover, imprecise estimation of non-specific binding result usually in concave Scatchard plots (Kermode, 1989).

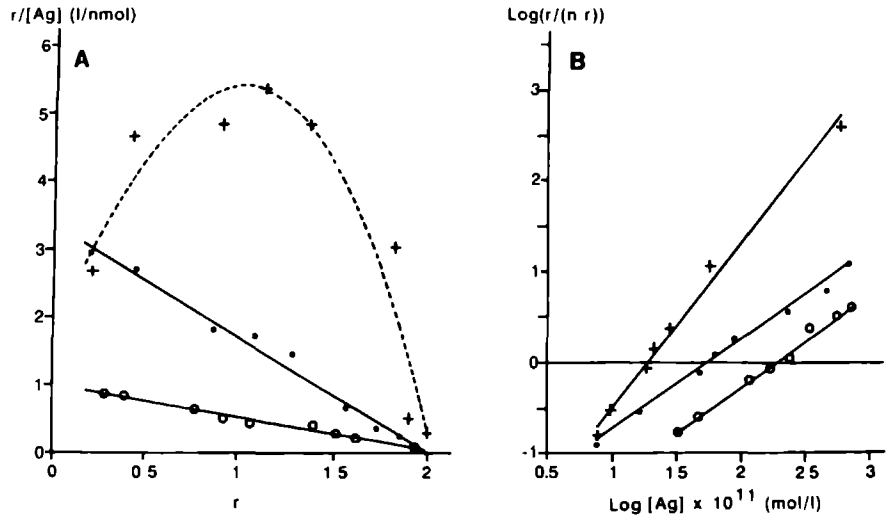

Fig. 4. Scatchard plot (A) and Sips plot (B) of hCG binding to MAb OT-7B (●); OT-9A (o) and OT-3A (+) at RT. The MAb concentrations were 7 nM, 7.5 nM and 7 nM, respectively. Numerical values for K_a and α are given in Table III.

Inadequate separation. In order to achieve adequate separation in 5 min of bound and free Ag, an amount of 2.4 mg DASP was used (see Fig. 2). Especially in the case of convex Scatchard plots the amount of DASP added to the same Ab concentration was increased. However, no significant decrease in convexity was observed. The heterogeneity index (α) using 2.4 mg DASP was 1.7 ± 0.1 and for 6 mg DASP 1.65 ± 0.1. Also after substituting RaM DASP by GaM Fc specific, the convexity remained unchanged.

Disturbance of the equilibrium. Dissociation of bound Ag from the Ab-Ag complex during separation was investigated by increasing the DASP incubation time. No differences in K_a values and convexity could, however, be observed for up to a 30 min DASP incubation time. The results are shown in Table II.

Reproducibility of the Procedure using SPIA

The reproducibility and precision of the method at RT was tested by repeated determination of the antibody affinities. The mean affinity of several anti-hCG MAbs were calculated from five or six independent determinations. Each test being performed on a different day and in triplicate. The overall results are summarized in Table III.

Table II

Disturbance of the equilibrium during the DASP incubation period

DASP[a] Incubation time (min)	MAb OT-7B Sips[b] $K_a \times 10^9$ (l/mol)	MAb OT-7B Index (α)	MAb OT-3A Sips[b] $K_a \times 10^9$ (l/mol)	MAb OT-3A Index (α)
2	1.80	1.03	N.D[c]	N.D[c]
5	1.73	1.01	4.7	1.65
10	1.79	0.97	5.1	1.72
15	1.68	1.01	4.7	1.80
30	1.83	1.04	4.8	1.68

a 2.4 mg of DASP was used and each hCG concentration was tested in triplicate.
b Results are based on equation (3) and linear regression analysis.
c not determined.

Table III

The reliability and reproducibility of the procedure using SPIA

MAb	No. measure-ments[a]	conc. (nM)	Scatchard[b] $K_a \times 10^8$ (l/mol)	Sips[b] $K_a \times 10^8$ (l/mol)	Index (α)
OT-7A	5	20	0.06 ± 0.01	0.06 ± 0.01	0.98 ± 0.04
OT-6A	5	20	0.10 ± 0.02	0.10 ± 0.02	1.01 ± 0.06
OT-7B	6	7	18 ± 1	18 ± 1	1.00 ± 0.03
OT-9A[c]	5	7.5	4.8 ± 0.4	5.1 ± 0.4	1.02 ± 0.04
OT-3A	6	7	N.D.[d]	48 ± 4	1.75 ± 0.1

a Each measurement was performed in triplicate.
b The results represent the mean value ± standard deviation of the number of measurements and are based on linear regression analysis. (regression coefficients were 0.99).
c Equilibrium binding analysis were performed in 0.15 M Tris/HCl, 0.2 M NaCl, 4 g/l BSA, 0.1 g/l Thiomersal pH 7.4.
d not determined.

Discussion

The data presented in this paper demonstrate the reliability and reproducibility of the technique for the determination of antibody affinities in the range of 10^6 - 10^{10} l/mol. The values of K_a determined by the method using SPIA are similar to the values obtained by a competitive RIA. Moreover, the measured affinity constants of MAbs against hCG are in the range ($1x10^7$ - $4x10^{10}$ l/mol) currently described in the literature (Ehrlich et al., 1982; Berger et al., 1984; Singh and Singh, 1985).

This technique has a number of advantages compared with other commonly used techniques. First, no labelling of either antibody or antigen are required and the initial antigen-antibody reaction is carried out in solution. This means no alteration of antibody affinity due to labelling or immobilization. Furthermore, the SPIA agglutination procedure is easy to perform (one step assay) and has a low coefficient of variation (≤ 5%) (Leuvering et al., 1980a).

The occurrence of convex Scatchard plots (cooperativity) for some individual anti-hCG MAbs in solution is remarkable. Theoretical analyses have indicated that a number of experimental artefacts may explain the non-linear Scatchard plots. Recently Kermode (1989) has described various artefacts together with their usual effects on Scatchard plots. He stressed that essentially all binding studies are affected by some or all of these described artefacts. The magnitude of their impact on the Scatchard plots, however, differs considerably from one study to another.

The study reported here excludes a significant contribution of the described potential artefacts that cause non-linearity of the Scatchard plots and demonstrates that the interactions of some MAbs are highly ("positive") cooperative.

The phenomenon of cooperative interactions has already been described for antisera (Matsukura et al., 1971; Weintraub et al., 1973) and mixtures of MAbs directed against hCG and other antigens (Tosi et al., 1981; Ehrlich et al., 1982 and 1983). However, the mechanisms proposed by these authors with respect to positive cooperative interactions are not directly applicable to our binding studies in which we use one particular MAb directed against one single antigenic site on hCG.

The mechanism of the apparent cooperative interaction has been investigated (Chapter 3).

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CHAPTER 3

AFFINITY OF MONOCLONAL ANTIBODIES; INTERPRETATION OF THE POSITIVE COOPERATIVE NATURE OF ANTI-hCG/hCG INTERACTIONS

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Abstract

The interaction of some individual MAbs and human chorionic gonadotrophin (hCG) showed apparent positive cooperativity as observed by equilibrium binding studies. This form of cooperative interaction has now been further characterized. The main results were: (1) the apparent positive cooperativity was strongly dependent upon concentration and temperature; (2) the cooperativity was strongly reduced by using peptic $F(ab')_2$ fragments of IgG and became undetectable when the MAb was replaced by its F(ab) fragment; (3) the molecular mass of the complex changed from 226 kDa to 450 kDa upon increasing hCG/MAb ratio. From these and additional results it is hypothesized that the apparent positive cooperativity results from self (Fc-Fc) associations mediated or facilitated by prior antigen binding.

Introduction

The development of hybridoma antibody technology (Köhler et al., 1975) has provided monoclonal antibodies (MAbs) that bind to single antigenic determinants. However, a critical factor with respect to the usefulness of a MAb is the affinity constant (also called antibody affinity), that characterizes the interaction between the MAb and its corresponding antigen (Ag) (Metzger et al., 1984). The expression of antibody affinity is largely controlled by the variable domains on heavy and light chains, which determine the strength of the interaction (Mariuzza et al., 1987). However, the hinge region and the CH1 domain, which are believed to be responsible for interdomain flexibility (Schneider et al., 1988; Burton, 1990), may also influence the affinity of an antibody.

Recently, a method using SPIA was developed for the determination of affinity constants for MAbs in buffered solution and without labelling of either MAb or Ag (Van Erp et al., 1991). In these equilibrium binding studies, it was demonstrated that (murine IgG_1) MAbs specific for hCG can bind in a "positive cooperative" way. This phenomenon of binding enhancement, which could not be ascribed to artefacts in the procedure (Van Erp et al., 1991), has been further characterized.

In this report some aspects will be described to support a role for the Fc and hinge region in the cooperative binding behaviour of (some) MAbs directed against hCG. The mechanism for cooperative antibody binding, which is hypothesized to result from self association mediated or facilitated by prior Ag binding, is to be distinguished from previously described mechanisms for cooperative binding of two MAbs of distinct specificity for hCG (Weintraub et al., 1973; Ehrlich et al., 1982; Moyle et al., 1983).

Materials

MAbs directed against hCG were produced and characterized as described previously (Van Erp et al., 1991). Highly purified hCG (12 100 IU/mg) and normal mouse IgG were obtained from Diosynth (The Netherlands) and Nordic (The Netherlands) respectively. Molecular weight standards for HPSEC (thyroglobulin, 669 000; ferritin, 440 000; IgG, 150 000; BSA, 67 000; ovalbumin, 43 000 and chymotrypsinogen-A 25 000) were obtained from Pharmacia (Uppsala, Sweden). $F(ab')_2$ specific GaM IgG was purchased from Cappel. All other chemicals were of analytical reagent grade quality.

Papain digestion and purification of F(ab) fragments

Buffer exchanges of the MAb solutions were performed on a column of Sephadex G-25 (PD10, Pharmacia) with 0.2 M sodium acetate buffer, pH 5.5. These solutions were diluted to an IgG concentration of 5 mg/ml. Subsequently, EDTA and cysteine were added to the final concentrations of 1 mM and 50 mM respectively. Digestions were performed using 1:100 (w/w) ratio of papain (type III, Sigma) to IgG at 37°C. The progress of digestion as a function of time was monitored by HPSEC using a Zorbax GF-250 column (Dupont) equilibrated with a 0.2 M sodium phosphate buffer pH 7.0 as eluent. After incubation, the Fab fragments were purified by Sephacryl S-200 HR (Pharmacia) gel filtration (0.15 M Tris/HCl buffer pH 7.6), followed by protein A-Sepharose CL-4B (Pharmacia) affinity chromatography. The purity of the fragment was subsequently checked by HPSEC and SDS-PAGE.

Preparation of $F(ab')_2$ fragments

Buffer exchanges of the MAb solutions were performed on a PD10 column with 0.1 M sodium acetate, 0.07 M sodium chloride, pH 3.5. Pepsin (3 100 U/mg, Sigma) at 1 mg/ml in the same acetate buffer was added to the antibody solution to give a pepsin-antibody ratio of 1:100 (w/w). After an incubation for 2 h at 37°C, the digestion was stopped by adjusting the pH of the reaction mixture to pH 8.0 with 1.5 M Tris (about 1/10 volume of protein solution). The $F(ab')_2$ fragments were subsequently purified by gel filtration on Sephacryl S-200 HR followed by protein A-Sepharose CL-4B affinity chromatography and characterized as described in the section above.

Protein A affinity chromatography

Samples, diluted with an equal volume of binding buffer (1.5 M glycine, 3 M NaCl, pH 8.9), were applied to a 5 ml protein A-Sepharose CL-4B column (Pharmacia) which was equilibrated with the same binding buffer. The flow rate was 0.8 ml/min. The effluent was monitored at 280 nm and binding buffer was passed until no more protein was eluted from the column. Buffer exchanges of the non-bound protein fraction in the effluent was subsequently performed on a PD10 column (Pharmacia) with 0.2 M sodium phosphate buffer pH 7.0. The bound protein fraction was desorbed from the column using a stepwise pH gradient of 100 mM citrate buffer (Tijssen, 1985).

Determination of antibody affinity

The affinity of a MAb directed against hCG was determined as described previously (Van Erp et al., 1991). In this procedure the Ab-Ag complexes in the equilibrium mixtures were separated from free Ag by a rabbit anti-mouse (RaM) immunosorbent (DASP). In the case of binding studies with IgG fragments, the separation of bound and free Ag was achieved using an excess of DASP/$F(ab')_2$ specific goat anti-mouse IgG.

HPSEC analysis of Ab/Ag mixtures

A constant amount of monomeric anti-hCG MAb (0.8 μM) was incubated with highly purified hCG at various concentrations in 0.21 ml of 0.2 μm filtered 0.2 M sodium phosphate buffer, pH 7.0. After incubation at RT for 2 h, 50 μl were injected onto Superose 6 (Pharmacia) and Biosil TSK 250 (Bio-Rad) columns, which were equilibrated with the same phosphate buffer. The flow rate used was 1 ml/min and the effluent was monitored at 206 nm. The identity of peaks was established using a calibration curve of molecular mass standards.

Protein determinations

The protein concentrations were determined by measuring the absorbance at 280 nm and using extinction coefficients of 1.45 $(cm \cdot mg/ml)^{-1}$ for IgG, $F(ab')_2$ and Fab, and 0.39 $(cm \cdot mg/ml)^{-1}$ for highly purified hCG (Van Erp et al., 1991). The molecular masses of IgG, $F(ab')_2$ and Fab were taken as 150, 100 and 50 kDa, respectively. A molecular mass of 38 kDa was used for hCG (Moyle et al., 1983).

Calculations

Calculations and interpretation were based on Scatchard (1949) and Sips (1948) plots. The curve fitting (third order polynomial fit) used for non-linear Scatchard plots represents the simplest way to acquire qualitative characterization of binding and was chosen to demonstrate the likelihood of apparent positive cooperativity. Real K_a values can only be derived from linear Scatchard plots or Sips plots (least square regression).

Results

Concentration dependence of cooperativity

The interaction of some MAbs and hCG showed apparent positive cooperativity as observed by equilibrium binding studies (Table I). Additional experiments using anti-hCG MAb OT-3A and OT-0A demonstrated that this phenomenon was dependent on MAb concentration. As illustrated in Fig. 1, the Scatchard plot for MAb OT-3A showed a convex curve, characteristic of positive cooperativity, when the MAb concentration was about 7 nM at RT. At a MAb concentration of 1.4 nM, a progressive decrease in the convexity of the curve was observed. In addition, the Sips analysis of the MAb OT-3A binding data was consistent with the findings of a concentration dependent cooperative interaction as shown in Table II.

Table I

Affinity values (K_a) and heterogeneity indices (α) of MAbs directed against hCG obtained from Sips Analysis

MAb	Sips[a] $K_a \times 10^8$ (l/mol)	Heterogeneity Index (α)
OT-7A	0.06 ± 0.01	0.98 ± 0.04
OT-6A	0.10 ± 0.02	1.01 ± 0.06
OT-7B	18 ± 1	0.98 ± 0.05
OT-4E	52 ± 4	0.99 ± 0.06
OT-3A	48 ± 4	1.75 ± 0.10
OT-0A	55 ± 6	1.31 ± 0.07

[a] The results represent the mean value ± SD of at least three experiments and are based on linear regression analysis.

Table II

The relationship between MAb concentration and heterogeneity index (α) obtained from Sips analysis

MAb OT-3A [nM]	normal mIgG [nM]	Index[a] (α)
7	-	1 7 ± 0 1
1 4	-	1 1 ± 0 1
1 4	5 6	1 1 ± 0 1

[a] *The results represent the mean value ± SD of three experiments and are* based on linear regression analysis

In order to demonstrate that this phenomenon was due to interactions between specific anti-hCG MAbs and not a characteristic of general mouse IgG per se, various concentrations of hCG were incubated with 1.4 nM MAb OT-3A adjusted to 7 nM with normal mouse IgG. The Scatchard plot obtained was subsequently compared with the Scatchard plots for MAb OT-3A at concentrations of 1.4 nM and 7 nM However, no increase of convexity was observed by the addition of normal mouse IgG (see Fig. 1)

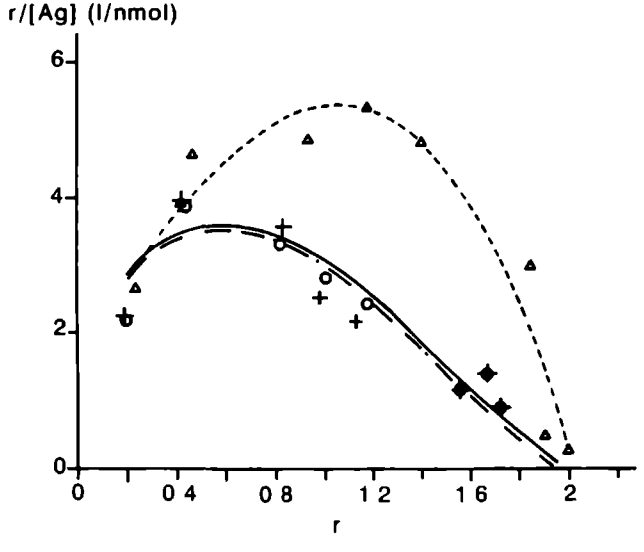

Fig 1 Scatchard plot of MAb OT-3A at different antibody concentrations, 7 nM (Δ), 1 4 nM (o) and 1 4 nM adjusted to 7 nM with normal mouse IgG (+)

Molecular mass of the hCG/anti-hCG complex

The preceding observations strongly suggest a concentration dependent association between anti-hCG MAbs mediated or facilitated by prior Ag binding (Steiner, 1980; Na et al., 1985). Therefore, equilibrium binding studies of MAb OT-3A and OT-0A were performed on both a Superose 6 and a Biosil TSK 250 column in order to determine the molecular mass of the complex using molecular mass calibration curves. When analyzed separately, IgG and hCG eluted as single peaks with apparent molecular

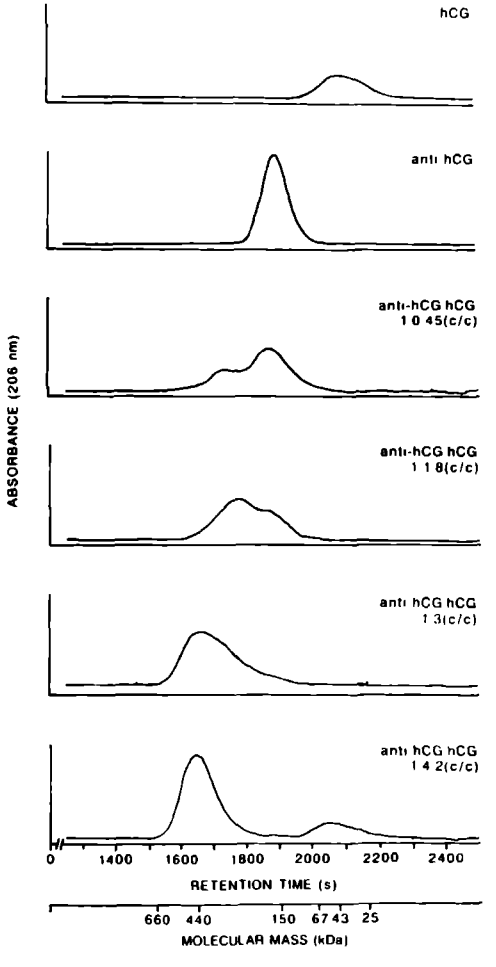

Fig. 2. Chromatograms of anti-hCG/hCG mixtures obtained using a Superose 6 HPSEC column (c/c; concentration ratio). The absorbance at 206 nm is plotted against retention time. The apparent molecular masses were estimated using a calibration curve.

masses of 160-170 kDa and 50-60 kDa respectively. The deviation for hCG from the value of 38 kDa may have been due to the ≈30% carbohydrate content of hCG (Shimohigashi et al., 1983). With increasing saturation of the Ab, the Ab-Ag complexes eluted with increasing molecular masses up to ± 450 kDa on both gel filtration columns. The results obtained with the Superose 6 column are represented in Fig. 2.

Since a molecular mass of 450 kDa is significantly different from the expected (theoretical) Ab-Ag complex of 226 kDa (IgG 150 kDa; hCG 38 kDa), it is unlikely that this shift in apparent molecular mass on both columns is mediated entirely by the carbohydrate content of hCG. In addition, control experiments using MAb OT-6A (no cooperative binding behaviour) resulted in an apparent molecular mass of ± 240 kDa for the Ab-Ag complex at an MAb/hCG concentration ratio of 1:8.

The effect of antibody fragmentation

In order to examine the effect of the antibody structural integrity on the occurrence of "cooperativity", fragments of the MAb OT-3A were prepared. Preparations of IgG, $F(ab')_2$ and Fab were subsequently incubated with hCG at comparable concentrations and equilibrium binding analyses were performed in triplicate as described in the Materials and Methods section. The apparent cooperative interaction of anti-hCG IgG was strongly reduced when $F(ab')_2$ fragments were used and was no longer detected

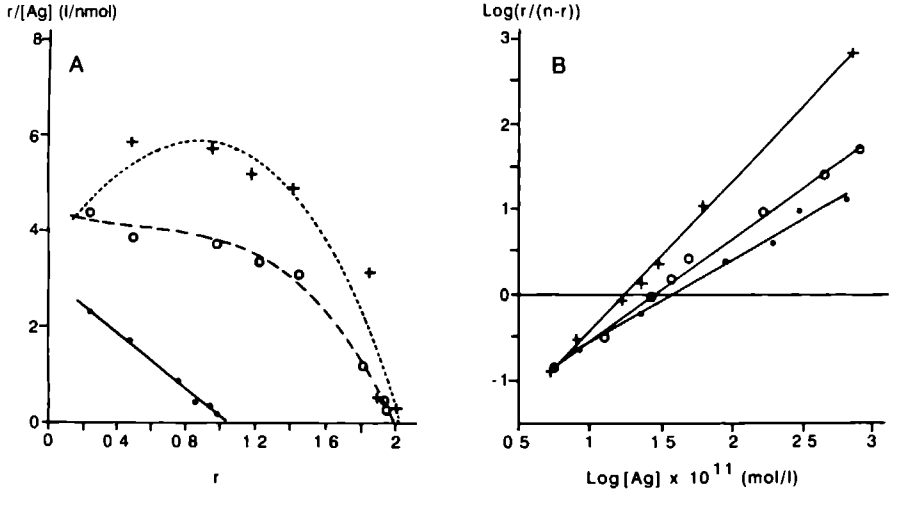

Fig. 3. Scatchard plot (A) and Sips plot (B) of hCG binding to MCA OT-3A IgG (+), $F(ab')_2$ (o) and Fab (●). The concentrations were 7.5 nM; 7 5 and 9 0 nM respectively. The heterogeneity indices (α) for MAb OT-3A IgG, $F(ab')_2$ and Fab fragments were· 1 77 ± 0 09, 1 21 ± 0.07 and 0 99 ± 0.04, respectively

when the antibody was replaced by its Fab fragment (see Fig. 3).

The same concentration dependence of the convexity of the Scatchard plots as found for IgG was observed for MAb OT-3A $F(ab')_2$ fragments. Therefore, it is suggested that both the Fc region and, to some extent, the hinge region participate in the association of IgG molecules. Another possible explanation for the weak cooperative binding of the $F(ab')_2$ fragments, namely binding site heterogeneity, is unlikely because of the homogeneous Fab interaction. Allosteric cooperative interactions are unlikely since these interactions should be independent of fragment concentration.

The effect of temperature on apparent positive cooperativity

The intensity of the cooperative binding of hCG with MAb OT-3A was also strongly influenced by temperature. At 0°C, the interaction of MAb OT-3A (5.5 nM) with hCG showed strongly convex Scatchard plots ($\alpha >> 1$) indicating highly cooperative binding. As illustrated in Fig. 4, the heterogeneity index (α) at 0°C was 1.62 ± 0.12 for MAb OT-3A. At higher temperatures, the apparent positive cooperativity decreased and the Scatchard plots became less convex. At 35°C, the heterogeneity index decreased to 1.17 ± 0.05. Similar results were obtained for a lower concentration of MAb OT-3A (Fig. 4) and for other anti-hCG MAbs OT-0A and OT-1C.

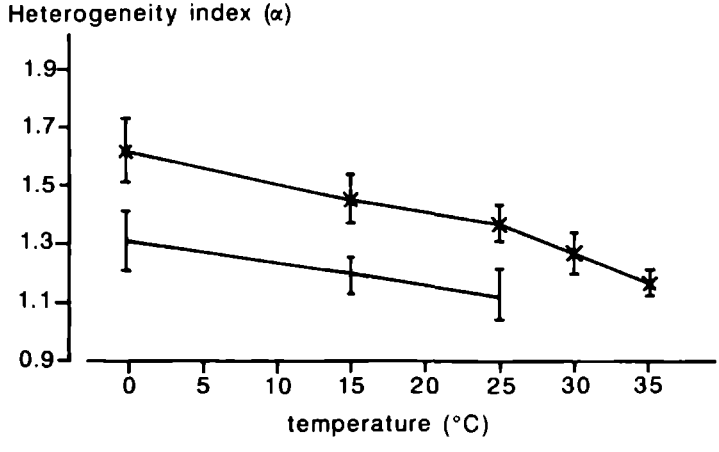

Fig. 4. The relationship between the temperature and the heterogeneity index (α) of MAb OT-3A binding with hCG. The time to reach equilibrium was determined previously.(20 h at 0 °C and 3 h for the other temperatures). The MAb concentrations were 1.4 nM (●) and 5.5 nM (x). The data are expressed as means of triplicate determinations.

Discussion

It was recently reported that hCG and some individual anti-hCG MAbs can exhibit evidence of apparent positive cooperative binding (Van Erp et al., 1991).

The present study demonstrated that the apparent positive cooperative interaction of an individual MAb directed against (a single epitope on) hCG was markedly dependent upon concentration. The convexity of the Scatchard plot, as illustrated for MAb OT-3A (Fig. 1), decreased with decreasing MAb concentration and the heterogeneity index (α), which is taken as a measure of apparent cooperativity, changed from 1.7 at a concentration of 7 nM to 1.1 at 1.4 nM. This dependence of convexity upon concentration is a characteristic phenomenon for associating systems (Steiner, 1980; Na et al., 1985). A concentration dependent association between anti-hCG MAbs, which is mediated or facilitated by prior combination with antigen is proposed because addition of normal mouse IgG to 1.4 nM MAb OT-3A did not increase the convexity of the almost linear Scatchard plot.

The results obtained using both a Superose 6 and Biosil TSK 250 column for anti-hCG MAbs OT-3A and OT-0A were consistent with this hypothesis. The molecular mass of the hCG/MAb complex changed from 226 kDa to 450 kDa upon increasing hCG/MAb ratio. This apparent molecular mass of 450 kDa for the hCG/MAb complex is significantly different from the expected $(hCG)_2$-MAb complex of 226 kDa (IgG, 150 kDa; hCG, 38 kDa) and provides substantial support for an associating system which might account for the cooperative nature of binding.

The MAb OT-3A derived $F(ab')_2$ fragments still exhibited some cooperative binding but these fragments were not as effective as the intact MAb. If the MAb OT-3A was replaced by univalent Fab fragments the phenomenon of cooperative binding was no longer detectable. This suggests that the Fc region and possibly the hinge region of the IgG molecule contribute to the association mechanism. Moreover, the homogeneous interaction between Fab fragments and hCG makes Ag-Ag association as an explanation for the cooperative binding behaviour unlikely.

Moreover, the demonstration that the cooperative interaction of hCG with MAb OT-3A increased at decreasing temperatures, can be explained by non-covalent Fc-Fc interactions, the strength of which increase with decreasing temperature.

All of the present observations are in agreement with the proposed hypothesis of a concentration dependent non-covalent Fc-Fc interaction between anti-hCG MAbs, mediated or facilitated by prior antigen binding. However, the mechanism of the increase of affinity due to Fc-Fc interactions remains to be elucidated. Possible explanations might be: (1) conformational changes due to association; (2) association resulting in a local increase of Ab binding sites which may decrease the dissociation

rates and thereby increase the affinity constant characterizing the binding of MAb to its Ag

The described cooperative interaction between one particular MAb and hCG is to be distinguished from the previously described mechanisms for cooperative binding of two MAbs having distinct specificities for hCG (Ehrlich et al , 1982) Nevertheless, the latter investigations did indicate that Fc regions might play a role in cooperative anti-hCG/hCG interactions. Finally, it may be noted that the phenomenon of Fc-Fc interactions has also been observed by Moller (1979), Greenspan et al (1988) and Werthén et al (1988)

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CHAPTER 4

MONITORING OF THE PRODUCTION OF MONOCLONAL ANTIBODIES BY HYBRIDOMAS. PART I: LONG-TERM CULTIVATION IN HOLLOW FIBRE BIOREACTORS USING SERUM-FREE MEDIUM

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O.T. Schönherr and T.C.J. Gribnau

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Abstract

The long-term cultivation of hybridoma cells in hollow fibre bioreactors using serum-free medium, was monitored with respect to quantitative and qualitative aspects of the produced MAbs, cell viability, LDH and proteolytic activity. During the culture periods of hybridoma cells producing MAb OT-1C and 3A, the MAb concentration showed a decreasing trend with a concomitant increase of IgG fragments. The major IgG fragments did not bind the antigen and the molecular masses were significantly different from the corresponding IgG heavy and light chains. In addition, a good correlation was found between cell lysis, the presence of acid protease(s) and IgG fragments. The physicochemical and immunochemical properties of the "intact" MAbs (such as molecular mass, IEF patterns and affinity) did not change significantly during the culture period.

Introduction

The hybridoma technology (Köhler and Milstein, 1975) has provided a means of generating virtually unlimited quantities of monoclonal antibodies (MAbs). The traditional method for producing monoclonal antibodies is by growing hybridoma cells in the peritoneal cavity of histocompatible mice. However, apart from being "animal-unfriendly" and therefore less desirable, this method is uneconomical and not practical for the production of gram quantities or more. Therefore, several *in vitro* methods applying hollow fibre cartridges (Hopkinson, 1985; Altshuler et al., 1986), porous beads (Posillico, 1986), stirred bioreactors (Lebherz, 1987) and ceramic matrices (Putnam, 1987) are currently used for large scale culture of hybridomas. The antibody yields for these culture systems depend upon the type of growth medium used (Rupp and Geyer, 1984), environmental factors (Lavery et al., 1985) and the production system itself (Seaver, 1987).

Recently, the *in vitro* production of MAbs against human chorionic gonadotrophin (hCG) using a hollow fibre dialysis module was described (Schönherr et al., 1987). In this system mouse hybridomas were grown to high cell densities ($>10^8$ cells per ml) in the extra-capillary compartment of dialysis modules with a constant supply of protein-free medium through the hollow fibres. The MAb production could be maintained for several weeks and MAbs were harvested in a concentrated form, free from serum components.

In-process control of the extracellular (micro)environment is, however, an inherent difficulty with (all) high density cell culture systems. The principal reason for this is that

the densely packed cells are exposed proportionally to a smaller volume of medium at one time. This may cause impairment of appropriate pH control and the depletion of nutrients or accumulation of waste products. Extreme (micro)environmental conditions can lead to cell death and cell lysis. Proteases or other components, such as glycosidases, released after cell lysis may subsequently lead to modification or even degradation of the desired MAb product (Moellering et al., 1990).

The objective of the present work was to monitor the MAb production in a hollow fibre bioreactor under serum-free conditions, in order to investigate the qualitative and quantitative aspects of MAb production in terms of physicochemical and immunochemical characteristics. One aspect of particular concern was the possible occurrence of product degradation by proteases or other components released after cell lysis.

Materials and Methods

Materials

Highly purified hCG (12 100 IU/mg) was obtained from Diosynth, The Netherlands. Molecular weight standards for HPSEC and SDS-PAGE were obtained from Pharmacia and Bio-Rad, respectively. Bovine spleen cathepsin D (13.5 U/mg protein; haemoglobin as substrate) was obtained from Sigma. All other chemicals were of analytical reagent grade quality.

Hybridomas

The development of hybridomas, producing MAbs directed against hCG, has been described elsewhere (Schönherr and Roelofs, 1982). The hybridomas producing the MAbs OT-1C and OT-3A (both directed against hCG and of the mouse IgG1 subclass) were used in this particular study.

Cell culture

The production of MAbs in serum-free medium using hollow fibre bioreactors was performed as described previously (Schönherr et al., 1987). Hybridomas were initially grown in roller bottles containing FCS (10%) supplemented culture medium (1:1 mixture of DMEM and Ham F12, Gibco) with the following additives: 2500 mg/l sodium bicarbonate, 2.3 mg/l 2-mercaptoethanol, 55 mg/l sodium pyruvate, 360 mg/l

L-glutamine, $4x10^{-4}$ mg/l sodium selenite, 1.22 mg/l ethanolamine, 62.5 mg/l streptomycin and 22 mg/l neomycin.

Cells from roller bottle cultures in the log-phase were inoculated in the extra-capillary compartment of the hollow fibre bioreactor (Nephross type Andante, Organon Teknika, The Netherlands) at a concentration of 5×10^6 cells per ml. The serum and protein-free medium was circulated through the fibres at a flow rate of 0.6 l/min while pH, temperature, O_2 and glucose consumption were monitored.

After an incubation period of two weeks, supernatant from the extra-capillary compartment was harvested twice a week and the viable and dead cell concentrations in each harvest were determined by trypan blue exclusion. In order to enable simultaneous assessment of other relevant parameters, the harvests were centrifuged and the supernatants divided in aliquots and stored at -20°C. Samples for the LDH activity measurements were stored at -70°C to prevent loss of LDH activity (Decker and Lohmann-Matthes, 1988)

HPSEC analysis

HPSEC was performed on a Zorbax GF-250 column (Dupont) using a 0.2 M NaH_2PO_4/K_2HPO_4 phosphate buffer pH 7.0 as eluent. The flow rate was 1 ml/min and 20 μl of a hybridoma culture sample (1:20 diluted with eluent) was injected after prior 0.2 μm filtration. The effluent was monitored at 206 nm as a function of time and the identity of peaks was established using a calibration curve of MW standards. The percentage of monomeric IgG was calculated using the formula:

$$\frac{A_{mon} \times 100\%}{A_{total}} = \% \text{ monomeric IgG} \qquad (1)$$

where A_{mon} = peak area of monomeric IgG and A_{total} = total area above baseline.

Determination of MAb concentration

SPIA

The total mouse IgG concentration in samples of hybridoma cultures was determined using the SPIA agglutination procedure as described elsewhere (Leuvering et al., 1983). For this purpose the gold sol particles were coated with rabbit anti-mouse immunoglobulin. To avoid sample interference in SPIA, cell-free samples and samples containing 10% FCS were diluted 1:10 and 1:50, respectively. The assays were performed in 96-well microtitration plates, and the extent of agglutination was

determined by measuring the absorbance at 540 nm after a 30 min incubation at RT using a Titertek Twinreader. The IgG concentration was calculated from a standard curve of each particular MAb.

HPSEC

A standard curve was prepared for each particular type of MAb by measuring IgG peak height from HPSEC chromatograms obtained by injecting 20 μl of samples containing various concentrations of protein A purified IgG. After HPSEC analyses of hybridoma culture samples (see above), the MAb concentrations were calculated from the corresponding linear standard curve.

Electrophoretic techniques and protein blotting

SDS-PAGE

Electrophoresis was performed under non-reducing and reducing conditions according to the method of Laemmli (1970). After gel electrophoresis, proteins were stained with Coomassie Brilliant Blue G-250 or transferred electrophoretically to a nitrocellulose membrane using a Bio-Rad trans-blot cell (Towbin et al., 1979). The nitrocellulose membrane was blocked during 2 h at RT in 5 mM PBS containing 5% (v/v) horse serum (Gibco, The Netherlands) and 4% (w/v) bovine non-fat dry milk (Elk, DMV Campina B.V., The Netherlands). Subsequently, HRP conjugated anti-mouse IgG was added to the buffer and incubated for another 1 h. The nitrocellulose membrane was rinsed twice with PBS-Tween followed by two washes with 5 mM PBS. Bands were visualized using the following substrate solution: 2 ml 0.3% chloronaphtol in methanol mixed with 10 ml 5 mM PBS and 5 μl 20% H_2O_2.

IEF

The isoelectric points of the MAbs were determined by isoelectric focusing on Ampholine PAG plates (pH 3.5-9.5, Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Quantitation of LDH activity in culture supernatants

A modification of the method described by Decker and Lohmann-Matthes (1988) was used to determine the enzyme activity in each harvest. Samples (50 μl) of a hybridoma culture medium or fresh medium (blank) were pipetted into 96-well

microtitration plates. Subsequently, 100 μl of 0.2 M sodium phosphate buffer pH 7.5 containing 1.8 mM sodium pyruvate (Sigma) was added. After agitation and incubation at 37°C, the enzymatic reaction was started by the addition of 50 μl freshly prepared NADH solution with an absorbance at 340 nm of 4.0 (Sigma, preweighted vials, 2.5 mg/ml phosphate buffer). The decrease in absorbance at 340 nm was measured with 60 s intervals using a Titertek Twinreader. The LDH activity was calculated according to the formula:

$$\text{LDH activity} = \frac{(A_{340nm}) \times V}{\epsilon_{NADH} \times t} \quad (\mu mol/min) \qquad (2)$$

where $(A_{340nm}) = A_{340nm}(t=0) - A_{340nm}(t=4 \text{ min})$, pathlength 1 cm; V = volume (ml); t = time (min) ; ϵ_{NADH} = extinction coefficient of NADH at 340 nm.

Proteolytic activity

Protease substrate gel tablets (Bio-Rad), containing agar and bovine casein, were used for the determination of proteolytic activity. The manufacturer's procedure was followed with some modifications. Two substrate gel tablets were resuspended in 10 ml 0.15 M sodium acetate buffer pH 5.0 and the suspension was heated subsequently in a boiling water bath for 3 min. The viscous liquid was cooled down to 55°C and was pipetted onto a plastic diffusion plate. Sample wells were punched in the gel and were filled with 15 μl hybridoma cell culture supernatant. Buffer or fresh medium were used as negative controls. The plates were incubated for 16 h at 37°C. The transparency of the rings was enhanced by overlaying the plates with 3% (v/v) acetic acid. After 2 h the diameters of the rings were measured. The proteolytic activities were calculated from a pure protease standard curve.

Determination of antibody affinity

The affinity of MAb OT-1C and OT-3A was determined and calculated as described elsewhere (Van Erp et al., 1991a).

Results

Cell viability and LDH activity

The viable and dead cell concentrations in each harvest taken during the cell culture period of MAb OT-1C are shown in Fig. 1A. After inoculation of approx. 5 x 10^6 cells per ml culture medium, the viable cell concentration exhibited a slight increase followed by a period of decrease over the next 20 days. This was caused by the change of the culture

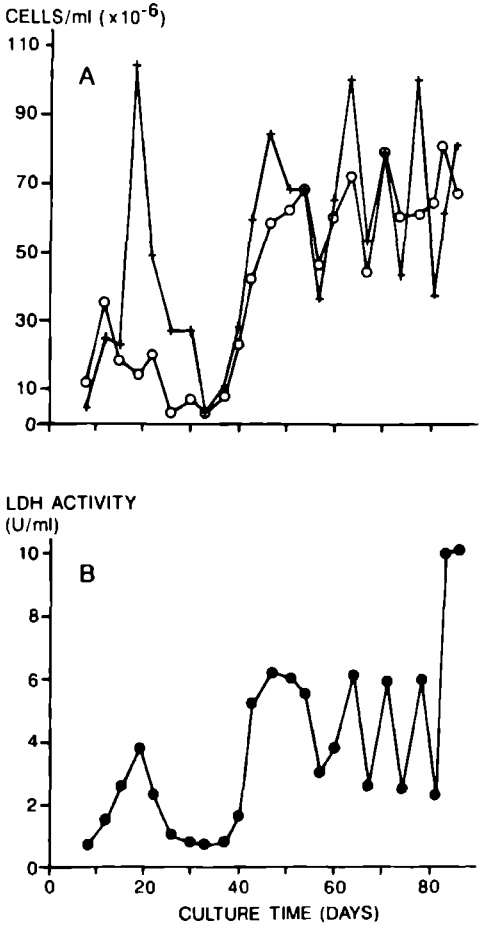

Fig. 1. Long-term cultivation of hybridoma cells under serum-free conditions using hollow fibre bioreactors. (A) Concentration of viable (o) and dead (+) cells in the harvests taken during the culture period of MAb OT-1C. (B) LDH activity profile of the culture medium during the same culture period.

in the extra-capillary compartment from a serum-containing to a serum-free medium. Subsequently, a period of exponential growth was observed until the concentration of viable cells reached a relatively constant value. Apart from the fluctuations, the concentration of viable cells remained at this relatively constant value for the whole culture period. The concentration of dead cells increased rapidly after inoculation and reached a maximum value after 20 days followed by a period of decline. The dead cell concentration showed about the same trend as the viable cell concentration, starting from day 33 until the end of the culture. The pattern of viable and dead cells during the culture was the same for cells producing OT-3A and OT-1C.

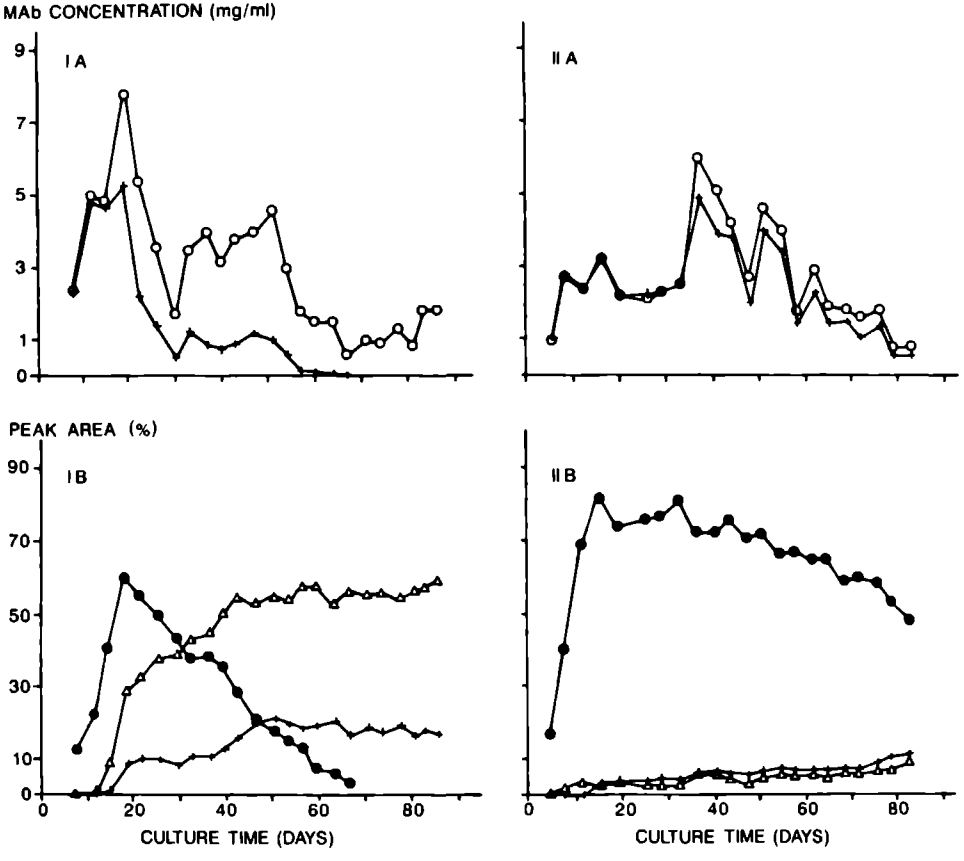

Fig. 2. Monitoring of MAb OT-1C (I) and OT-3A (II) production. (A) IgG concentrations during the culture period as measured by SPIA (total mouse IgG: o) and HPSEC (monomeric IgG: +). (B) The percentages of the major components found in the harvests by HPSEC analysis: monomeric IgG (o); component A (Δ) and component B (+).

The presence of intracellular LDH in the culture medium is considered as an indication of cell lysis (Petersen et al., 1988). The LDH activity profile during the cell culture period of MAb OT-1C is presented in Fig. 1B. The increase in LDH correlates well with the cell death observed. A quantitative comparison of LDH release with cell death characteristics would require the total number of dead cells to be determined. However, both the heterogeneity of the culture system and the occurrence of cell lysis make it impossible to calculate the total number of dead cells.

Monoclonal antibody production

The antibody production during the cell culture period of MAb OT-3A and OT-1C was monitored by measuring the MAb concentration in each harvest using both the SPIA agglutination procedure and HPSEC. The results are shown in Fig. 2A. After an initial increase in MAb concentration, a decreasing trend in concentration was observed for both cell cultures.

HPSEC analyses showed that the decrease of the concentration of intact MAb was accompanied by an increase of two quantitatively major components (A and B) with molecular masses of 47 kDa and 25 kDa, respectively (see Fig. 2B). Both phenomena occurred to a much larger extent for MAb OT-1C than for MAb OT-3A. These results were confirmed by SDS-PAGE (Fig. 3). Based on this technique, the molecular masses of component A and B were 43 kDa and 23/21 kDa, respectively.

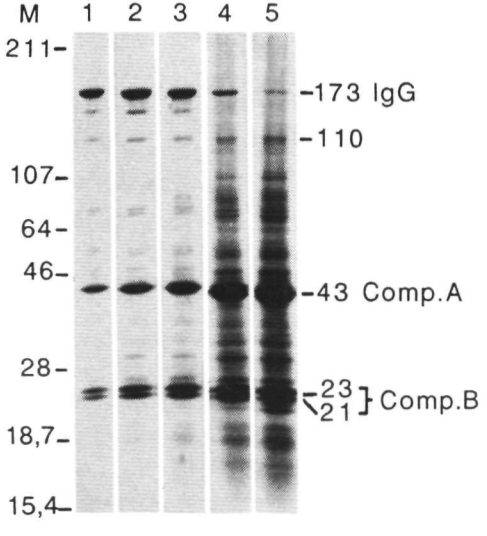

Fig. 3. Non-reducing SDS-PAGE of samples taken during the culture period of MAb OT-1C using a 4-26 % gradient gel. The samples applied to the gel had similar mouse IgG concentrations based on SPIA values. Lane 1-5 contained samples taken on day 19, 33, 40, 60 and 81, respectively. The molecular masses (kDa) of the marker proteins are shown at the left while the calculated values (kDa) of the IgG and IgG fragments are presented at the right position.

Immunoblotting with HRP labelled anti-mouse IgG showed that both the major components (A and B) and also some minor fragments of 110 kDa, 36 kDa and 30 kDa were of mouse IgG origin (Fig. 4).

The cascade of weakly stained bands present just below the major antibody band (173 kDa), which are also reactive with anti-mouse antibodies, are generally thought to be glycosylation variants since these bands were also found in highly purified MAb solutions and for IgG from ascites. The molecular masses of the two major IgG fragments (A and B), are significantly different from the corresponding IgG heavy (50 kDa) and light (25/26 kDa) chains. Furthermore, fragment A was reduced by the addition of ß-mercaptoethanol demonstrating the presence of disulphide bonds (data not shown).

The presence of mouse IgG fragments in cell culture supernatants might explain the discrepancy between the MAb concentrations determined by SPIA and HPSEC (Fig. 2, I/IIA). In contrast with the HPSEC method used, the SPIA procedure measures the total mouse IgG concentration, including possible fragments.

Proteolytic activity

The results described in the section above suggest that the antibodies produced by hybridomas in hollow fibre bioreactors undergo (some) degradation during the culture period, possibly as a result of proteolytic enzymes released from dead or lysed cells.

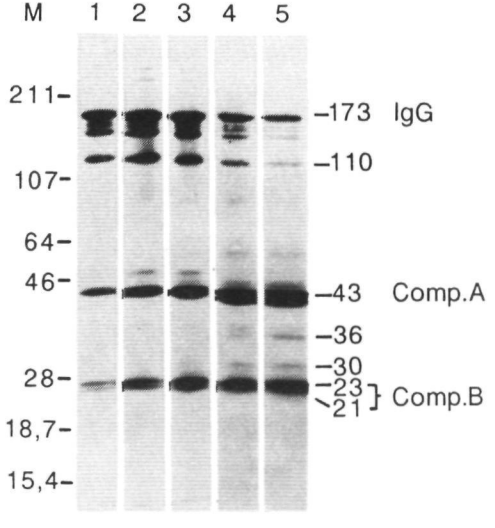

Fig. 4. Western blot after non-reducing SDS-PAGE (4-26% gradient gel) of samples taken during the culture period of MAb OT-1C. For more details see legend of Fig. 3.

Therefore, the presence of proteolytic enzymes in the culture medium was investigated and it appeared that cell-free supernatants from serum-free hollow fibre cultures contained a proteolytic activity which appeared to be similar to that of lysosomal cathepsin D (Van Erp et al., 1991c). In order to quantify this proteolytic activity during the culture periods, a standard curve of bovine spleen cathepsin D was used. The profiles obtained of the proteolytic activity are illustrated in Fig. 5.

Comparison of the proteolytic activity profile with both the LDH activity (Fig. 1B) and the fragmentation profile (Fig. 2, I/IIB) demonstrated that the increase in LDH activity is accompanied by an increase of proteolytic activity followed by the formation of IgG fragments.

Monoclonal antibody characterization

Affinity

The affinities of the MAbs directed against hCG (OT-1C and 3A) were determined during the culture period. HPSEC experiments demonstrated that the two major IgG fragments (A and B) did not bind hCG which was also confirmed by equilibrium binding

Table I

Comparison of affinity values obtained for MAb OT-1C and OT-3A during the culture period

Harvest (days)	MAb[a] OT-1C		MAb[a] OT-3A	
	$K_a \times 10^9$ (l/nmol)	Index[b] (α)	$K_a \times 10^9$ (l/nmol)	Index[b] (α)
18	1.5	0.98	4.1	1.28
28	1.4	1.00	3.9	1.33
43	1.5	0.99	3.8	1.23
55	1.5	1.04	4.1	1.25
66	1.5	1.05	4.1	1.35
76	N.D.[c]	N.D.[c]	4.0	1.28
83	N.D.[c]	N.D.[c]	3.9	1.30

[a] The (Intact) MAb concentration was 3.5 nM based on HPSEC values.
[b] Index of heterogeneity (α) obtained from Sips analysis of the binding data.
[c] not determined.

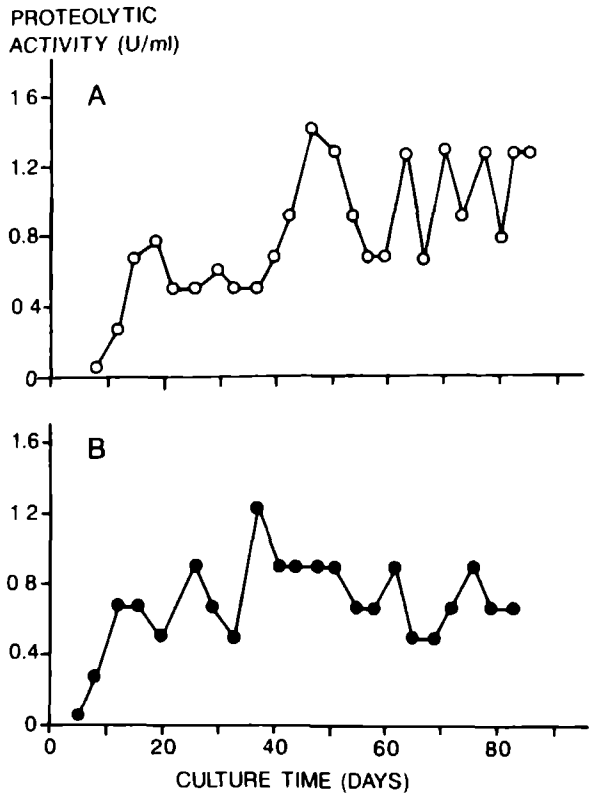

Fig. 5. Proteolytic activity profiles during the culture periods of MAb OT-1C (A) and OT-3A (B). The acid protease activity is expressed as equivalent units of cathepsin D from standard plots.

analysis (data not shown). The K_a values for MAb OT-1C and 3A during the culture period were obtained from Sips analysis of binding data as described elsewhere (Sips, 1948; Van Erp et al., 1991a,b) and remained at a constant value (Table I). No K_a values are presented for MAb OT-1C after 66 d, since no more intact IgG could be detected (HPSEC) at that stage. The index of heterogeneity (α) calculated from the Sips plot deviates from 1 in the case of MAb OT-3A. An explanation for this phenomenon was described recently (Van Erp et al., 1991b).

Isoelectric focusing

Samples taken from each harvest were analyzed using IEF. The IEF patterns obtained after staining and immunoblotting with HRP conjugated anti-mouse IgG were subsequently compared with protein A purified IgG standards. No major differences

occurred in the IEF patterns during the culture period. The IEP values for MAb OT-1C and MAb OT-3A ranged from 6.2-6.9 and 7.2-8.1, respectively.

Discussion

Monitoring of both MAb OT-1C and 3A production using hollow fibre bioreactors showed a decreasing trend in MAb concentrations during several culture periods. Additional experiments indicated that the decrease of the concentration of intact MAb was accompanied by an increase of the concentration of IgG fragments. This effect was much larger for MAb OT-1C than for MAb OT-3A.

Further analysis of the major IgG fragments (A and B) obtained for MAb OT-1C and 3A demonstrated that (a) the major fragments had no binding activity for hCG, (b) their molecular masses were different from the corresponding IgG heavy and light chains and (c) the IgG fragment A could be reduced by ß-mercaptoethanol. On the other hand, characterization of the "intact" MAbs OT-1C and 3A showed no significant differences in molecular mass, affinity and IEF patterns over the course of the cultures.

The concentration of dead cells and LDH activity in the medium determined during the culture period correlates well and indicated a trend of increasing cell lysis. This increase in cell lysis was accompanied by the increase of a proteolytic activity which resembled the lysosomal cathepsin D action (Van Erp et al., 1991c).

The correlation found between cell lysis, proteolytic activity and the occurrence of IgG fragments suggests that the MAbs undergo some degradation as a result of proteolytic enzyme(s) released from dead or lysed cells.

Alternatively, lysed cells may have caused release of retained antibody fragments. However, additional experiments showed no significant increase of antibody or fragments immediately upon lysis of both viable and dead cells. The production of incomplete IgG by the hybridoma cells, similar to that observed for some myeloma cell lines (Coffino and Scharff (1971); Cotton et al., 1973), might be considered as another possibility for the occurrence of IgG fragments. However, the loss of production in myeloma cell lines was seen as a loss of heavy and light chains. Since the molecular masses of the major IgG fragments observed were different from the corresponding IgG heavy and light chains, this possibility seems unlikely. Several authors reported recently (Frame and Hu, 1990; Heath et al., 1990; Ozturk and Palsson, 1990) that a loss of antibody productivity in long-term cultivations of hybridoma cells was due to the occurrence of a nonproducing subpopulation of cells.

In spite of the above uncertainties, (a) proteolytic enzyme(s) seem(s) to be a significant factor in MAb degradation during long-term cultivation of hybridoma cells under serum-free conditions using hollow fibre bioreactors. Therefore, the acid proteolytic activity has been further characterized and the influence on MAb degradation was investigated more thoroughly. The results are described in part II of this study (Van Erp et al., 1991c/Chapter 5).

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CHAPTER 5

MONITORING OF THE PRODUCTION OF MONOCLONAL ANTIBODIES BY HYBRIDOMAS. PART II: CHARACTERIZATION AND PURIFICATION OF ACID PROTEASES PRESENT IN CELL CULTURE SUPERNATANT

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Abstract

An acid proteolytic activity has been found in cell culture supernatants from long-term cultivations of hybridoma cells in hollow fibre bioreactors using serum-free medium. The proteolytic activity has now been further characterized and the main results were: (1) the proteolytic activity showed a maximum around pH 3 and declined essentially to zero at pH 8; (2) the activity was specifically inhibited by pepstatin A; (3) the acid proteases consisted of two sets of closely spaced bands with apparent molecular masses of 40-45 kDa and 90-105 kDa respectively; (4) the protease bands (40-45 kDa and 90-105 kDa) were reactive with anti-human cathepsin D; (5) the IEP values of the acid proteases ranged from pH 4.55-6.5. Furthermore, IgG incubation with the acid proteases isolated from hybridoma cells yielded fragments similar to those found in serum-free hollow fibre cell culture supernatants. These results indicated that the IgG fragments are the result of degradation by cathepsin D like proteases released after cell death or cell lysis.

Introduction

Monoclonal antibodies (MAb) have been widely used for many purposes in various fields. The increasing demand for MAbs has led to the development of several high density culture systems for the industrial scale production of MAbs (Hopkinson, 1985; Altshuler et al., 1986; Posillico, 1986; Putnam, 1987). Each method, however, provides a different (micro)environment for the hybridoma cells, an environment which ultimately determines the productivity of the system. Therefore, large scale production of MAbs requires knowledge of the environment of hybridoma cells for optimization of culture conditions and process control.

Recently, the monitoring of large scale production of MAbs under serum-free conditions using hollow fibre bioreactors was described (Van Erp et al., 1991). The results suggested that the MAbs produced by hybridomas in hollow fibre bioreactors over a period of several weeks, undergo (some) degradation, probably as a result of proteolytic enzymes released from dead cells. However, the degree of degradation was different for each particular MAb. Therefore, MAb degradation by the acid protease(s) observed has been further investigated.

In this report, the characterization and purification of the acid proteases will be described. Furthermore, the IgG fragments obtained by incubation with both the purified proteases and bovine cathepsin D were compared with the IgG fragments found in serum-free hybridoma cell culture supernatants.

Materials and Methods

Materials

Antiserum raised in rabbits against human cathepsin D was kindly provided by Dr. G.J. Strous (Laboratory of Cell Biology, University of Utrecht, The Netherlands). Purified bovine spleen cathepsin D (13.5 U/mg protein; haemoglobin as substrate), Pepstatin A , Leupeptin and soy bean trypsin inhibitor were purchased from Sigma. Molecular weight standards for HPSEC and SDS-PAGE were obtained, respectively, from Pharmacia and Bio-Rad. Anti-rabbit IgG alkaline phosphatase conjugate and BCIP/NBT color development substrate were purchased from Promega. All other chemicals were of analytical reagent grade quality.

Hybridomas and cell culture

The preparation of hybridomas producing MAbs directed against hCG and the production in hollow fibre bioreactors under serum-free conditions was described elsewhere (Schönherr and Roelofs, 1982; Schönherr et al., 1987). The hybridomas producing mouse MAb OT-1C and OT-3A (both directed against hCG and of IgG1 subclass) were used in this study.

The SPIA agglutination procedure

The total mouse IgG concentration in hybridoma cell culture supernatants were determined using the SPIA agglutination procedure as described previously (Van Erp et al., 1991).

Electrophoretic techniques

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970). For the electrophoretic analysis of proteases, the polyacrylamide gels contained 1 mg/ml casein and were prepared and used essentially as described by Heussen and Dowdle (1980). After electrophoresis, the gels were washed twice for 30 min with 2.5% Triton X-100 in water to remove SDS and restore the enzyme activity. Subsequently, the gels were incubated overnight at RT in 0.15 M sodium acetate buffer pH 4.0 followed by staining for 3 h in a 0.1 % solution of Coomassie Brilliant Blue G250 (CBB) in

methanol:acetic acid:water (40:10:50). The gels were destained in the same solution without CBB G-250.

IEF

Isoelectric focusing was performed on Ampholine PAG plates (pH 3.5-9.5, Pharmacia, Uppsala, Sweden) as described in the instructions. After focusing, the gel was washed during 15 s in a 0.15 M sodium acetate buffer (pH 4.0). Subsequently, the gel was overlayed with a hot agar melt (50°C) containing a bovine casein suspension in 0.15 M sodium acetate buffer (pH 5.0). The agar/casein gel was incubated overnight at RT followed by staining with Serva violet (0.04%, w/v) according to the procedure described above.

Immunoblotting

After SDS-PAGE, proteins were transferred electrophoretically to a nitrocellulose membrane (Towbin et al., 1979). The nitrocellulose membrane was blocked for 3 h with 5 mM PBS containing 5% (w/v) non-fat dry milk (Elk, DMV, Campina B.V., The Netherlands). Subsequently, anti-human cathepsin D antiserum (1:100) was added to the buffer and the membrane was incubated for another 2 h. After two washes with PBS-Tween and PBS, the nitrocellulose membrane was incubated with alkaline phosphatase conjugated anti-rabbit IgG followed by another four washes. The bands were visualized using BCIP/NBT substrate.

Proteolytic activity

Protease substrate gel tablets (Bio-Rad) were used for the determination of proteolytic activity. The manufacturer's procedure was followed with some modifications as described previously (Van Erp et al., 1991). The conversion of specific activity values based on casein and haemoglobin substrates was performed using a standard curve of bovine cathepsin D in the casein assay.

Inhibitors of the acid proteases

A culture supernatant containing the acid proteases was incubated for 20 h at 37°C in the presence of inhibitors. Samples without inhibitors were taken as blank. After incubation the degree of inhibition was analysed using SDS-PAGE. The proteolytic activity of these samples was also measured by the casein protease assay (Bio-Rad).

Purification of the acid proteases

The acid proteases were purified from hybridoma cells according to the method of Huang et al. (1979) with some modifications.

Two hundred ml of a hybridoma cell pellet, obtained after harvesting of hollow fibre bioreactors, was resuspended in 200 ml of a 1 mM Na_2HPO_4 solution (4°C). During the following isolation procedures, 1 mM Na_2HPO_4 was included in all solutions to protect the acid proteases from phosphatase digestion (Nakao et al., 1984). The suspension was sonicated for 1 min at setting 4 of a Branson sonifier (B-12) and clarified by centrifugation at 10^5 N/kg for 30 min. The pH of the supernatant was subsequently adjusted to 3.7 with 4 M HCl and the precipitate was removed by centrifugation at 10^5 N/kg. To the acid supernatant, solid sodium sulphate was added to give a 25% (w/v) saturation. The precipitate, which contained the acid proteases was collected and a buffer change was made on a Sephadex G-25 column (Pharmacia) using the 1 mM Na_2HPO_4 solution as mobile phase. For the second time, the pH of the solution was adjusted to 3.7 with 4 M HCl and the precipitate formed removed by centrifugation. Buffer exchanges of the acid supernatant was performed on a Sephadex G-25 column with 0.05 M sodium phosphate buffer pH 7.4. The solution was subsequently subjected to a DEAE Sephadex A-25 (Pharmacia) column which was equilibrated with the same sodium phosphate buffer. The breakthrough fractions of the DEAE Sephadex A-25 column were combined and adjusted to pH 3.7 with 4 M HCl. Subsequently, the obtained solution was mixed with 5 ml pepstatin A agarose gel (Pierce) which had been washed with 0.05 M sodium acetate buffer pH 3.5 containing 0.2 M NaCl. The gel suspension was rotated overnight, after which it was packed in a C(10/10) column (Pharmacia) and washed with 0.05 M sodium acetate buffer pH 3.5 containing 0.2 M NaCl. The acid proteases were eluted with 0.05 M Tris/HCl buffer pH 8.6 containing 2 M NaCl.

HPSEC analysis

HPSEC was performed on a Zorbax GF-250 column (Dupont) using a 0.2 M NaH_2PO_4/K_2HPO_4 phosphate buffer pH 7.0 at a flow rate of 1 ml/min. A sample (20 μl) was injected after prior 0.2 μm filtration and the effluent was monitored at 206 nm. The identity of peaks was established using a calibration curve of molecular weight standards and the percentage of monomeric IgG was calculated as described before (Van Erp et al., 1991)

Effect of pH on the enzyme activity

A hybridoma culture supernatant was diluted to an IgG concentration of 0.5 mg/ml followed by a dilution (1:1) with 0.05 M citrate phosphate buffer at different pH values. After an incubation period of 5 h at 37°C, the samples were analysed using HPSEC. The activity was expressed as a percent of digested IgG in comparison to the monomeric IgG peak observed at t=0.

Proteolytic digestion of IgG

The acid proteases purified from lysed hybridoma cells (0.3 U) were mixed with 0.3 mg of MAb OT-1C in 0.75 ml of a 0.1 M citric acid buffer pH 5.0 and incubated at 37°C. Control experiments were performed by incubating MAb with bovine cathepsin D and without enzyme. Samples were taken during the incubation period and the digestion was followed by HPSEC.

Determination of protein concentration

Protein concentrations were calculated from the absorbance at 280 nm using an extinction coefficient of 1.45 $(cm \cdot mg/ml)^{-1}$ for IgG. The BCA protein assay (Pierce) as described by the manufacturer using 96-well microtitration plates and BSA as standard, was also used.

Results

Effect of pH on the proteolytic activity

The effect of pH on IgG degradation in serum-free culture supernatants was determined and compared with the pH dependence of bovine cathepsin D activity. The pH activity profiles are illustrated in Fig. 1. Highest activities were observed between pH 2.3 and 4 with a peak maximum at pH 3. Obviously, pH dependence of the proteolytic activity in hybridoma cell cultures and of bovine spleen cathepsin D are similar.

Effect of protease inhibitors

Inhibitors can be used to reduce the proteolytic degradation of IgG and to classify the proteases involved in the degradation. The effect of several inhibitors were

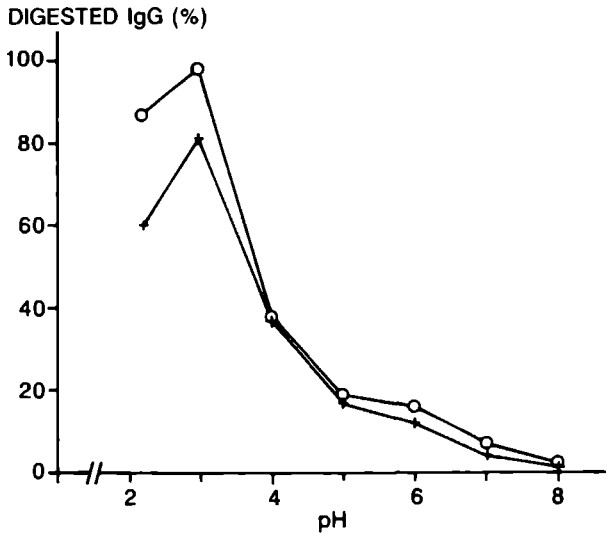

Fig. 1. pH activity profiles of both the proteolytic activity in hybridoma cell cultures (o) and bovine spleen cathepsin D (+) on IgG digestion.

Table I

Effects of inhibitors on the proteolytic activity in hybridoma cell culture supernatants

Inhibitor	Conc.	Inhibition (%)	
	(mM)	IgG degradation	Casein protease assay
Bacitracine	0.07	0	0
PMSF	10.0	0	0
EDTA	1.0	0	0
Leupeptin	0.06	0	0
$MgCl_2$	0.5	0	0
$CaCl_2$	0.5	0	0
Pepstatin A	0.018	100	100
Trypsin inhibitor	5 μg/ml	0	0
FCS	20 %	100	15

investigated on samples of hybridoma cell culture supernatants (MAb OT-1C and 3A) using SDS-PAGE and the protease substrate gel tablets (Bio-Rad). The results are presented in Table I and showed that the proteolytic degradation of IgG was only inhibited by pepstatin A (a carboxyl protease inhibitor) and by FCS at a relatively high concentration. The latter apparent inhibition of IgG degradation is most probably due to competition with an excess of protein substrate in FCS. Application of the casein protease assay (Bio-Rad) to the samples demonstrated that the proteolytic activity was only specifically inhibited by pepstatin A.

Electrophoretic analysis of the proteases

Electrophoretic analysis of four hybridoma cell culture supernatants on polyacrylamide gels containing casein, showed a significant protease activity after incubation at lower pH values. As can be seen from Fig. 2, the protease activity increased during the culture period and consisted of two sets of closely spaced bands at about 40-45 kDa and at about 90-105 kDa, respectively. Additional experiments (more sample and longer electrophoresis time) clearly showed that the high MW protease band consisted of three bands (Fig. 2, lane 5).

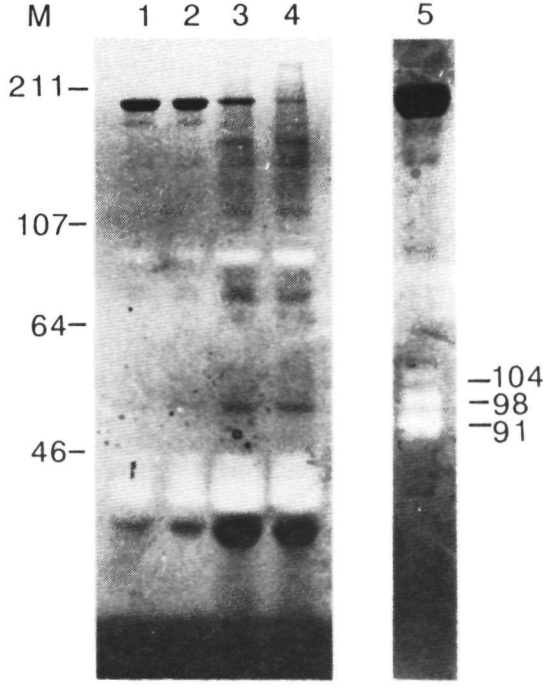

Fig. 2. Identification of proteases (light zones) under non-reducing conditions in 10% SDS polyacrylamide gels containing 1 mg/ml casein. Four samples taken during the culture period of MAb OT-1C were diluted to the same MAb concentration based on SPIA results. 25 µg of each sample was applied to the gel and electrophoresed during 4 h at 40 mA: lane 1-4, day 19, 40, 60 and 81 respectively. Lane 5; a concentrated sample (100 µg) of day 19 electrophoresed during 7 h. Marker proteins (kDa) are shown at the left while the calculated molecular masses (kDa) are shown at the right.

Immunoblotting with anti-human cathepsin D

Electrophoresis of cell culture supernatants followed by immunoblotting with rabbit anti-human cathepsin D antiserum (Rijnboutt et al., 1990) showed that both high and low MW protease bands were specifically stained (Fig. 3). From the Western blot it could also be obtained that the high and low MW proteases consisted, respectively, of three and two closely spaced bands.

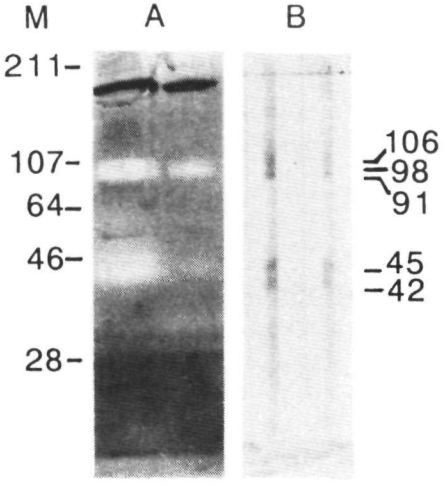

Fig. 3 A) SDS-PAGE of cell culture supernatant under non-reducing conditions in 10% SDS gel containing 1 mg/ml casein (Bio-Rad, Miniprotean II system). The light zones are areas of proteolytic activity. B) corresponding Western blot. Selective detection of cathepsin D was performed using rabbit anti-human cathepsin D. Molecular masses (kDa) of the marker proteins are shown at the left whereas the calculated values (kDa) are presented at the right position.

Isoelectric focusing

Isoelectric focusing showed that the IEP values of the acid proteases found in serum-free cell culture supernatant ranged from 4.55-6.5 (data not shown).

Purification of the acid proteases from hybridoma cells

Obviously, there are several similarities between the proteolytic activity found in cell culture supernatants and cathepsin D. Therefore, a purification procedure described for porcine cathepsin D was used to isolate the protease(s) from hybridoma cells (Huang et al., 1979). The scheme for this purification is illustrated in Table II and the experimental conditions are described in Materials and Methods. The inhibitor pepstatin A was used in the final affinity purification step. The elution profile of the pepstatin A agarose column (Pierce) is shown in Fig. 4. Analysis of enzyme activity using the casein protease assay

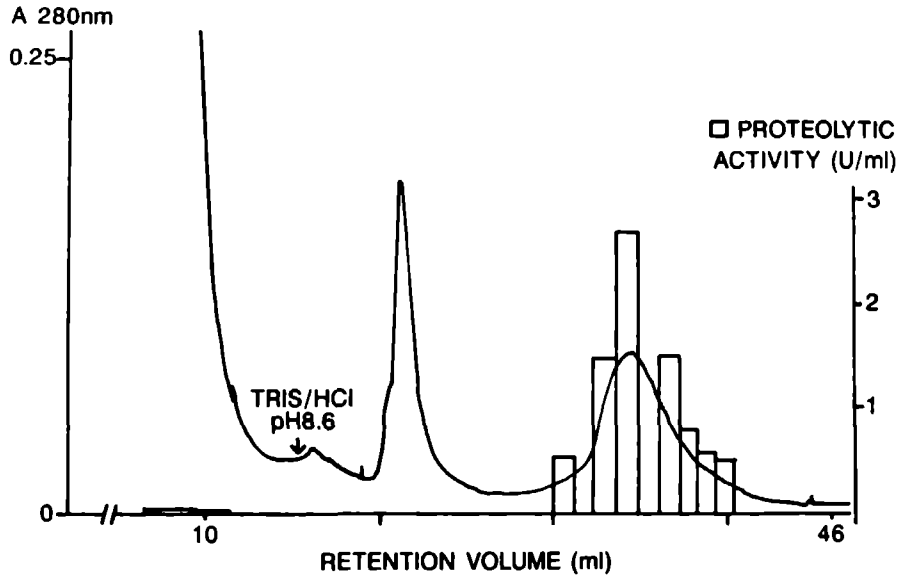

Fig. 4. Elution profile of the cathepsin D purification from hybridoma cells on a pepstatin A agarose column. The flow rate was 0.8 ml/min and the effluent was monitored at 280 nm. The enzyme activity in the desorption fractions is indicated by vertical bars. The fractions with missing vertical bars (peak 2) were not determined.

Table II

Purification scheme of the acid proteases from hybridoma cells

Purification steps	Total enzyme activity (U)[a]	Yield (%)
Homogenate	207	100
Supernatant	109	53
Acid supernatant I	96	46
Na_2SO_4 precipitate	88	42
Acid supernatant II	79	38
DEAE sephadex	57	28
Pepstatin A column	28	13

[a] One unit will produce an increase in A_{280} of 1.0 ml^{-1} min^{-1} at pH 3.0 at 37°C using haemoglobin as substrate.

(Bio-Rad) demonstrated that the second desorption peak contained most of the proteolytic activity.

On the other hand, the first desorption peak consisted of mainly nucleic acids as determined by both spectrophotometry (absorbance maximum at 259 nm) and agarose gel electrophoresis performed according to Maniatis et al. (1982). The fractions of peak 2 were pooled and the protein content was determined using the BCA protein assay. The total yield of enzyme from this purification procedure was 0.88 mg resulting in a specific activity of 31.1 U/mg protein.

SDS-PAGE of the purified enzyme(s) under non-reducing conditions showed two (broad) bands at about 40-45 kDa and about 90-100 kDa (Fig. 5). The two sets of closely spaced bands as observed in cell culture supernatant (using SDS-PAGE/casein, based on enzyme activity) could not be distinguished. A special note should be made on the apparent low recovery from the pepstatin A affinity column. This apparent loss of total activity is most probably due to the separation of "activator" proteins from "cathepsin D" during this purification step as described by Huang et al. (1979; 1980).

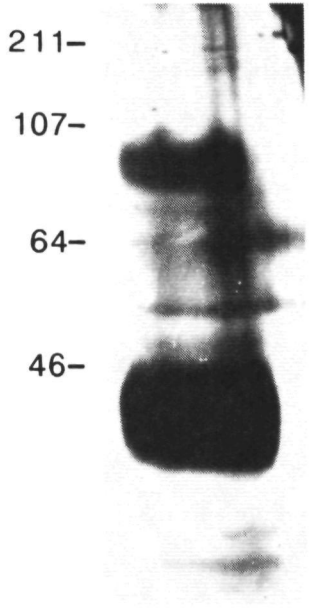

Fig. 5. SDS-PAGE of the purified proteases from hybridoma cells using a 10 % acrylamide gel. Proteins were stained with GELCODE Silver Stain (Pierce). Marker proteins (kDa) are presented at the left.

IgG digestion patterns

The purified proteases from hybridoma cells were incubated with IgG to confirm the findings of MAb degradation during production in hollow fibre bioreactors (Van Erp et al., 1991). Preliminary experiments demonstrated that below pH 5, the cathepsin D digestion is more rapid and leads to total degradation of the IgG and fragment proteins.

Therefore, the IgG digestion patterns of both the purified proteases and for comparative purpose, the commercially obtained bovine cathepsin D were investigated at a relatively mild acid condition of pH 5 to achieve a limited but detectable extent of digestion. The HPSEC elution profiles of the various digests are given in Fig. 6.

The digestion patterns obtained with the purified proteases and the bovine spleen cathepsin D (Sigma) are similar. Digestion of MAb OT-1C yields fragments with a molecular mass of approx. 110 kDa (D), 47 kDa (A), and 25 kDa (B). These fragments were similar to the IgG fragments found in serum-free hollow fibre cell culture supernatants (Fig. 6). In addition, the results obtained for OT-1C were found also for OT-3A but to a lower extent.

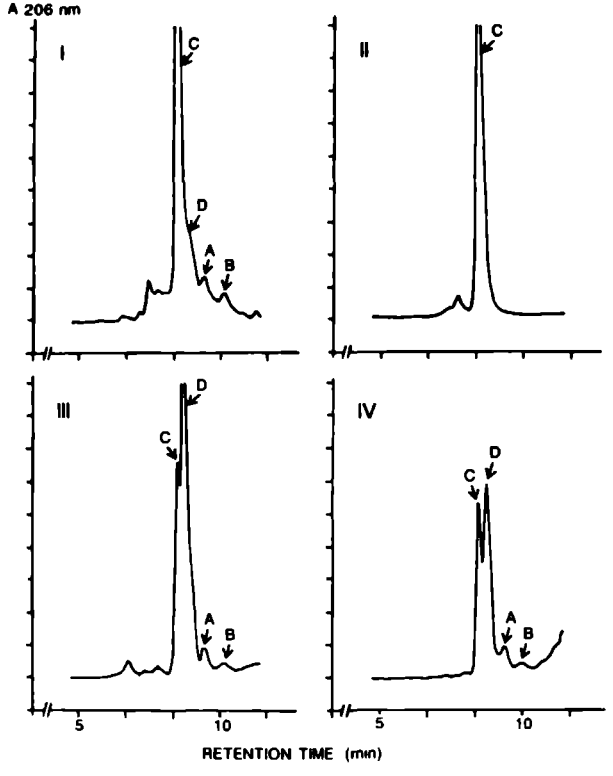

Fig. 6. HPSEC profiles of various MAb OT-1C digests obtained after mild treatment (pH 5) with purified cathepsin D at 37°C; I) a serum-free cell culture supernatant; II) Protein A purified MAb OT-1C with purified cathepsin D at t=0 and III) after t= 5 days For comparative purpose: IV) treatment with bovine spleen cathepsin D (t= 5 days). The absorbance is given in arbitrary units. Peak identification: C = IgG (165 kDa); D, A and B are IgG fragments with a molecular mass of 110, 47, and 25 kDa, respectively.

Discussion

There are indications that MAbs produced by hybridoma cells under serum-free conditions undergo proteolytic degradation (Schlaeger et al., 1987, Van Erp et al., 1991). This paper confirms and extends these results regarding both the occurrence of an acid proteolytic activity, its identity, and the influence on IgG degradation during long-term cultivation of hybridoma cells under serum-free conditions using hollow fibre bioreactors.

The acid proteases found in serum-free cell culture supernatants were specifically inhibited by the addition of pepstatin A indicating that the proteolytic enzyme(s) found in cell culture supernatants belong(s) to the carboxyl- dependent class of proteases (Huang et al. 1980). The effect of pH on IgG degradation demonstrated highest enzyme activities between pH 2.5 and 4.0. The enzyme activity was zero at pH 8. A similar activity profile was found for bovine spleen cathepsin D. Furthermore, cathepsin D from various tissue sources has been reported to have optimum activities between pH 2.8 and 4.0 (De Lumen and Tappel, 1970; Barret, 1977). Electrophoretic analysis of cell culture supernatants on gels containing casein demonstrated that the acid proteases consisted of two sets of closely spaced bands at 40-45 kDa and 90-105 kDa, similar to the protease bands described by Schlaeger et al. (1987).

In addition, immunoblotting with rabbit anti-human cathepsin D showed that both high and low molecular mass proteases were specifically stained and consisted, respectively, of three and two bands. Furthermore, isoelectric focusing of the cell culture supernatants showed that the IEP values of the proteolytic enzyme(s) ranged from 4.55-6.5, which is in reasonable agreement with values reported for cathepsin D from other species (Barret, 1977; Huang et al., 1979).

All of the present observations indicated that the acid proteases were similar to cathepsin D. The presence of proteases with molecular masses of 40-45 kDa and 90-105 kDa as found for hybridomas has also been described for cathepsin D from rat lymphoid tissue, mouse spleen (Yago and Bowers, 1975) and porcine spleen (Huang et al., 1979).

The described proteases were purified subsequently from hybridoma cells and incubated with IgG to confirm the degradation of MAbs as found for MAb OT-1C and OT-3A. Characterization of the purified cathepsin D by SDS-PAGE showed that both the 40-45 kDa and 90-100 kDa proteases were eluted from the pepstatin A affinity column. The degradation of MAb OT-1C, by the purified cathepsin D and bovine spleen cathepsin D both yields fragments with a molecular mass of approx. 110 kDa, 47 kDa and 25 kDa. The fragment with a molecular mass of 110 kDa is most probably the same as found by Schlaeger et al., (1987) who described that IgG molecules were digested resulting in $F(ab')_2$ like fragments.

During monitoring of MAb OT-1C and 3A production in hollow fibre bioreactors, IgG fragments with similar molecular masses were found although the fragment of 110 kDa was observed as a minor component (Van Erp et al , 1991) The quantitative differences between the amounts of each fragment found in digestion experiments and serum-free hollow fibre cell culture supernatants might be explained by differences in enzyme concentrations and environmental factors such as culture medium and pH, but is certainly also clone dependent On the other hand, the possibility that the hybridoma cells produce also non-intact IgG after a longer period is under investigation

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CHAPTER 6

THE EFFECT OF PURIFICATION METHODS ON MONOCLONAL ANTIBODY PROPERTIES

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submitted for publication

Abstract

Monoclonal antibodies directed against human chorionic gonadotrophin (hCG) were purified from serum-free hollow fibre cell culture supernatants. Starting with sodium sulphate fractionation as a pre-purification step, final purification was performed using Sephacryl S-300 HR gel filtration, Mono Q ion exchange - or protein A affinity chromatography. The effect of these methods of purification on the properties of the MAb products was investigated by several techniques. Special attention was also paid to the elimination of the acid proteases present in cell culture supernatant. The purity of the IgG solutions after sodium sulphate precipitation ranged from 86-90% whereas it was more than 95% for the chromatographically purified solutions. Protein A affinity chromatography, however, yielded MAb solutions showing light scattering, responsible for a reduced A_{280}/A_{254} ratio. In addition, MAb fractions free of the acid proteases were obtained only when ion exchange or protein A affinity chromatography were used as a polishing step. Equilibrium binding studies showed that the binding properties of the MAbs purified by ion exchange chromatography were (slightly) affected when compared to the otherwise purified MAb solutions. Finally, application of chromatographically purified IgG in a sandwich immunoassay for hCG yielded improved test performance as compared to the results obtained by sodium sulphate fractionated MAb fractions.

Introduction

Monoclonal antibodies (MAbs) are often applied in diagnostics to obtain more sensitive, specific and reproducible test systems. To control test development and production it is necessary to use well characterized (and purified) components of which MAbs present an important part.

MAbs can be readily obtained from hybridomas by *in vitro* cultivation (Köhler and Milstein, 1975) and a variety of methods are available for purification. The most frequently described methods involve salt fractionation, gel filtration, ion exchange and protein A affinity chromatography. These methods are used as either a single purification step or a successive two step combination of these methods (Goding, 1986; Manil et al., 1986; Tijssen, 1985; Duffy et al., 1989; Jiskoot et al., 1989; Perosa et al., 1990). Although a single purification step may yield sufficiently pure MAb solutions, the employment of two different purification techniques may be considered a prerequisite to obtain highly purified MAb solutions. The commonly used purification techniques encompass a wide range of chemical conditions (i.e. pH and ionic strength) which may

affect the properties of the MAb molecules (Underwood and Bean, 1985; McCue et al., 1988; Jiskoot et al., 1990).

In this paper the effect of particular purification methods on MAb properties was investigated for three different MAbs of two batches each. Special attention was also paid to the elimination of the acid proteases present in serum-free cell culture supernatants (Van Erp et al., 1991) by the particular method of purification. The properties of the MAb -IgG fractions were investigated by several techniques including UV/VIS spectrophotometry, high performance size exclusion chromatography (HPSEC), SDS-PAGE, isoelectric focusing (IEF), affinity measurements, protease assay, DNA assay and performance in a sandwich immunoassay for human chorionic gonadotrophin (hCG) using colloidal gold as label.

Materials and Methods

Materials

Three mouse monoclonal (anti-hCG) antibodies of the hybridoma cell lines OT-9A, 7B and 3A (two batches of each: I, II) were used in this study. The preparation of hybridomas and the production of the MAbs in hollow fibre bioreactors under serum-free conditions have been described elsewhere. (Schönherr et al., 1987). All MAbs are of the IgG1 subclass. The isoelectric points are: 7.2-8.1 (3A), 6.4-7.2 (9A) and 5.9-6.6 (7B). Highly purified hCG (12 100 IU/mg) was obtained from Diosynth, The Netherlands. Sephacryl S-300 HR, protein A-Sepharose CL-4B, Sephadex G-25 and the pre-packed Mono Q (HR 5/5) and Superose 12 (HR 10/30) columns were obtained from Pharmacia, Uppsala, Sweden. Bovine spleen cathepsin D (13.5 U/ml protein; haemoglobin as substrate) and calf thymus DNA were purchased from Sigma. All other chemicals used were of analytical reagent grade quality.

Purification of monoclonal antibodies

The MAbs were isolated, after prior sodium sulphate fractionation (S), by one of the following techniques:

G Sephacryl S-300 HR gel filtration
P Protein A-Sepharose CL-4B affinity chromatography
I Mono-Q ion exchange chromatography.

Pre-purification

Pre-purification of the MAbs was performed using sodium sulphate fractionation (200 g/l) followed by dialysis against 0.15 M NaCl. The IgG solutions obtained (10 mg/ml in 0.15 M NaCl) were 0.2μm filtered and used as starting materials (S) for final purification.

Gel filtration

The pre-purified IgG (100-125 mg) samples were applied onto a Sephacryl S-300 HR column (ϕ 26, h 900 mm) which was equilibrated with 0.15 M NaCl. The flow rate was 0.5 ml/min and the effluent was monitored at 280 and 254 nm. Fractions containing monomeric IgG were pooled.

Protein A affinity chromatography

The pre-purified IgG samples (100-125 mg) were diluted with an equal volume of binding buffer (1.5 M glycine, 3.0 M NaCl, pH 8.9) and applied to a Protein A-Sepharose CL-4B column (ϕ 16, h 95 mm), which was equilibrated with binding buffer. The flow rate was 1.2 ml/min. The bound fraction was desorbed from the column using 0.1 M sodium citrate buffer, pH 5.0. Column regeneration was performed with 0.1 M sodium citrate buffer, pH 3.0. Monitoring was performed with respect to absorbance (A_{280}), pH and conductivity. Buffer exchanges of the MAb solutions were performed on a Sephadex G-25 column with 0.15 M NaCl solution.

Ion exchange chromatography

The pre-purified IgG samples (100-125 mg) were diluted (1:20) using 0.02 M Tris/HCl buffer with a pH of 1.0 unit above the IEP of the particular MAb. The samples were applied at a flow rate of 1.0 ml/h to a Mono Q (HR 5/5) column equilibrated with the same buffer. Desorption was performed using a linear gradient of 0-0.3 M NaCl in 0.02 M Tris/HCl buffer during 35 min. The effluent was monitored at 280 and 254 nm. Fractions containing more than 97% monomeric IgG, based on HPSEC, were pooled. Buffer exchanges of the IgG fractions were performed on a Sephadex G-25 column equilibrated with 0.15 M NaCl solution.

Protein determinations

Protein concentration of the IgG solutions was determined by measuring the absorbance at 280 nm assuming an extinction coefficient of 1.45 $(cm \cdot mg/ml)^{-1}$.

HPSEC analysis

Samples of the purified MAb solutions (100μl) were injected at a flow rate of 0.5 ml/min onto a Superose 12 (HR 10/30) column equilibrated with 0.15 M NaCl solution. Detection was performed at both 206 and 280 nm. The purity was expressed as % monomeric IgG (peak area/ total peak area ; at 280 nm).

Electrophoretic techniques

SDS-PAGE

IgG samples in reducing and non-reducing buffer, were applied to a SDS polyacrylamide gradient gel (4-26%), using the discontinuous buffer system as described by Laemmli (1970). Staining was performed with Coomassie Brilliant Blue according to the method of Neuhoff et al. (1985).

Isoelectric focusing

The isoelectric points of the MAbs were determined by isoelectric focusing using Ampholine PAG plates (pH range; 3.5-9.5, Pharmacia) according to the manufacturer's procedure.

Spectrophotometry

Spectra of 1.0-1.5 mg/ml IgG solutions in 0.15 M NaCl were recorded from 200-650 nm, using a Pye Unicam UV/Vis 8745 scanning spectrophotometer (1 cm cuvette, bandwidth 0.5 nm).

Affinity determination

The affinity of the MAbs directed against hCG were determined as described elsewhere (Van Erp et al., 1991a).

DNA assay

The DNA content of the IgG samples was determined according to the method of Erwin (1981), using calf Thymus DNA as standard.

Protease assay

Proteolytic activity in the final IgG solutions was assessed at pH 5 using Protease substrate gel tablets (Bio-Rad). The manufacturer's procedure was followed with some modifications as described previously (Van Erp et al., 1991c).

Immunoassay

A sandwich immunoassay for hCG was performed using colloidal gold as label according to the principle as described by Leuvering et al. (1981 and 1983). Both solid phase coating (MAb OT-9A and 7B) and gold conjugate (MAb OT-3A) were prepared from the pre- and final purified IgG solutions. Urine samples (n = 120), negative for hCG in other two in house pregnancy tests, were tested.

Results

Purity of the IgG solutions

Chromatographic and electrophoretic techniques

Following MAb purification, the degree of purity of the IgG solutions was assessed by HPSEC, SDS-PAGE and IEF. The results obtained from HPSEC analyses are presented in Table I. The purity of the IgG solutions after sodium sulphate precipitation ranged from 86-90% and was more than 95% for the otherwise purified IgG solutions (except OT-9A-II P). These results were confirmed by SDS-PAGE under non-reducing conditions.

In addition, testing of antibody heterogeneity by IEF yielded no significant differences between the pre- and chromatographically purified IgG solutions with respect to pH range and number of distinct bands.

Table I

Summary of purity according to HPSEC,
and spectral data of the pre- and final purified MAb solutions

MAb	Code[a]	purity[b]	A_{280}/A_{254}	A_{330}
9A-I	S	88 6	2 1	0 093
	G	95 5	2 5	0 015
	P	96 8	2 3	0 029
	I	98 9	2 4	0 011
9A-II	S	85 7	2 3	0 043
	G	98 9	2 5	0 005(-)
	P	91 2	2 3	0 064
	I	99 5	2 4	0 013
7B-I	S	87 1	2 0	0 105
	G	98 3	2 5	0 008
	P	97 4	2 1	0 126
	I	95 8	2 4	0 008
7B-II	S	86 7	2 0	0 110
	G	98 6	2 5	0 000
	P	96 0	2 2	0 097
	I	98 4	2 4	0 017
3A-I	S	87 7	1 5	0 267
	G	100	2 5	0 007
	P	99 5	2 2	0 110
	I	95 7	2 5	0 017
3A-II	S	90 4	2 0	0 090
	G	98 1	2 5	0 010
	P	98 8	2 0	0 131
	I	96 1	2 5	0 015

[a] Codes are explained in the section Materials and Methods
[b] Purity is expressed as percentage of the total peak area

Spectrophotometry

Further examination of the purity of IgG solutions was performed using spectrophotometry. The principal difference observed spectrophotometrically between the IgG solutions was the ocurrence of light scattering. The phenomenon of light scattering is best demonstrated at higher wavelength (>320 nm) where no IgG absorption occurs (Fig. 1). The results obtained for the IgG samples after prior 0.2 μm filtration are summarized in Table I. Light scattering was observed for all S and P purified IgG solutions whereas the G and I purified materials were all clear. In addition, the A_{280}/A_{254} ratios (after prior 0.2 μm filtration) of the S and P purified IgG samples were consistently lower as compared to the otherwise purified materials.

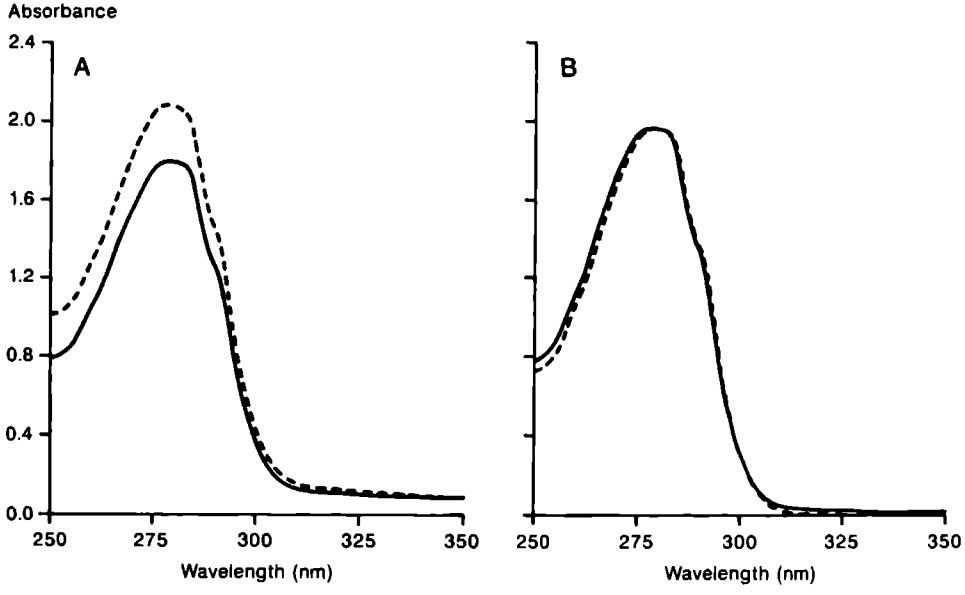

Fig. 1. UV spectra of MAb OT-7B-II solutions after: (A) sodium sulphate fractionation (---) and Protein A affinity chromatography (——), (B) gel filtration (---) and ion exchange chromatography (——).

Proteolytic activity

Recently, it has been demonstrated (Van Erp et al., 1991c,d) that serum-free cell culture supernatants contain (a) cathepsin D like acid protease(s) capable of degrading MAb molecules. The efficacy of each particular purification method in removing these proteases was investigated by measuring the acid proteolytic activity in the purified MAb solutions.

Table II

Proteolytic activity in pre- and final purified MAb solutions

MAb	Code[a]	Proteolytic activity	
		Batch I (U/ml)[b]	Batch II (U/ml)[b]
9A	C	0.8	0.6
	S	0.7	0.6
	G	0.4	0.3
	P	<[c]	<
	I	<	<
7B	C	0.7	0.8
	S	0.6	0.7
	G	0.3	0.4
	P	<	<
	I	<	<
3A	C	0.7	0.9
	S	0.7	0.8
	G	0.4	0.4
	P	<	<
	I	<	<

[a] C = cell culture supernatant; the other codes are explained in the section Materials and Methods.

[b] The acid protease activity is expressed as equivalent units of cathepsin D from standard plots.

[c] <; lower than detection limit (0.01 U cathepsin D/ml).

As shown in Table II , both sodium sulphate precipitation and gel filtration yielded IgG solutions which were still contaminated with the acid proteases. The latter, however, contained considerably less proteases as compared to the S purified materials. The MAb fractions purified by protein A affinity chromatography or ion exchange chromatography were free (lower than detection limit) from the cathepsin D like acid proteases.

Table III

Results of equilibrium binding studies of the MAbs with hCG

MAb[a]	Code[b]	Batch I		Batch II	
		K_a values[c] (l/nmol)	Index[c] (α)	K_a values[c] (l/nmol)	Index[c] (α)
9A	C	1.8	1.00	1.8	0.98
	G	1.7	0.98	1.7	1.05
	P	1.9	0.98	1.7	1.05
	I	2.2	1.17	2.0	1.15
7B	C	1.9	0.99	2.0	0.98
	G	1.9	1.00	2.0	1.03
	P	2.1	1.04	2.1	0.93
	I	2.7	1.25	2.1	0.96
3A	C	4.2	1.05	4.1	1.06
	G	4.2	1.05	4.3	1.00
	P	4.3	1.06	4.2	0.93
	I	4.7	1.39	4.4	1.18

[a] The MAb concentration used was 2 nM.
[b] C = cell culture supernatant; for the other symbols see section Materials and Methods.
[c] Calculations are based on the Sips equation.

Affinity measurements

In order to investigate whether changes (denaturation) in antigen binding activity had occurred during the purification procedures, the affinity of the MAbs in both cell culture supernatant and purified solutions were determined. The K_a values for the anti-hCG/hCG interaction were calculated using the Sips equation (Sips, 1948; Van Erp et al., 1991a,b) and are summarized in Table III. The K_a values obtained for the chromatographically purified MAb fractions were not substantially different from the values found for MAbs in cell culture supernatants. In addition, the heterogeneity indices (α) of the G and P purified MAb fractions (calculated from the Sips plot; Fig. 2) were equal to 1.0, indicating a homogeneous interaction between the MAb and Ag. The

heterogeneity indices of the Mab fractions obtained by ion exchange chromatography, however, were in general significantly higher than 1 (Fig. 2). This means that the MAb fractions were heterogeneous with regard to the affinity constant (K_a).

Performance in a sandwich immunoassay

The effect of the particular purification method on test performance of the monoclonal antibody was investigated using a sandwich immunoassay for hCG with colloidal gold as label (Leuvering et al., 1981 and 1983). Urine samples (n=120) negative in two other pregnancy tests were tested.

The assays based on S purified material showed two false positives (confirmed), which were negative (confirmed) in the assays based on the otherwise purified IgG. The latter assays gave no false positive results. Additionally, it was not possible to prepare a colloidally stable gold sol conjugate using the S purified MAb OT-3A fractions whereas stable conjugates were obtained from the chromatographically (P, I and G) purified MAb solutions.

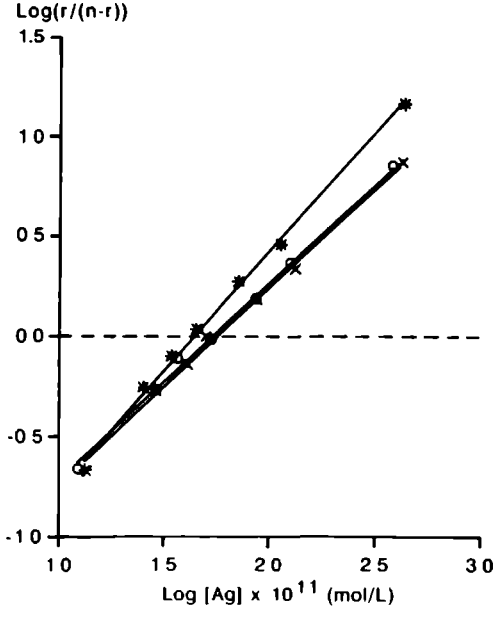

Fig. 2. Sips plots of the chromatographically purified MAb OT-9A-I; gel filtration (o), protein A affinity (x) and ion exchange chromatography (*).

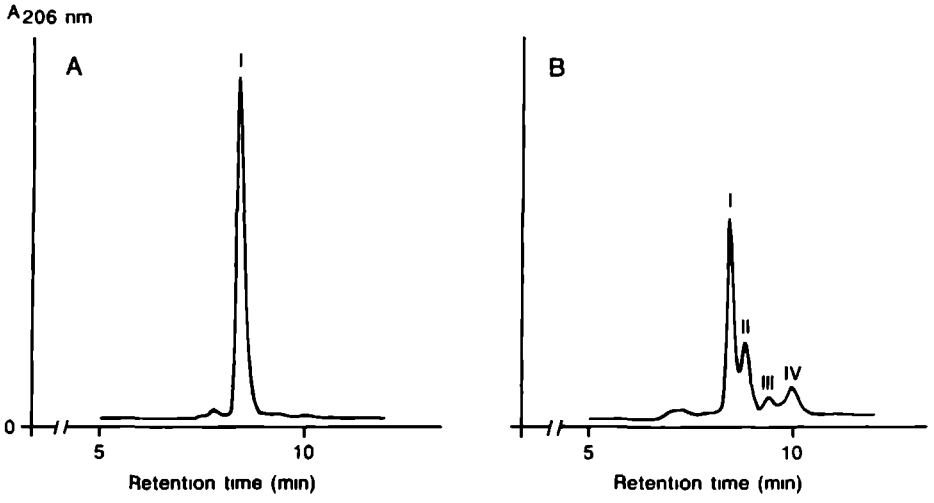

Fig. 3. HPSEC profiles of (A) MAb OT-3A directly after gel filtration (G); and (B) after storage for 7 month in 0.15 M Tris/HCl pH 7.2 containing 0 02% sodium azide at 37°C. HPSEC analyses were performed using a Zorbax GF-250 column (Dupont) as described elsewhere (Van Erp et al., 1991). Peak identification; I: IgG (165 kDa), II, III and IV are IgG fragments with molecular masses of 104 kDa, 45 kDa and 23 kDa respectively.

Discussion

In the present study, the effect of the particular purification method on MAb properties was investigated by a combination of several characterization methods.

The purity of the IgG solutions was examined under both native (HPSEC) and denatured (SDS-PAGE) conditions. Based on these two techniques, no substantial differences in purity (>95%, except 9A-II P) were observed for the chromatographically purified MAb fractions. The sodium sulphate purified IgG solutions (S), however, were considerably less pure (86-90%). Characterization of the purified MAb solutions by IEF, which has proven to be a valuable tool in the characterization of immunoglobulins (Hoffman, 1977; Hamilton, 1987), showed no significant changes in the IEF patterns.

Spectrophotometric analyses of the IgG solutions revealed both light scattering and a reduced A_{280}/A_{254} ratio for all S and P purified IgG fractions. The occurrence of light scattering for the S purified materials can be explained by the presence of aggregated IgG in the IgG fractions as determined by HPSEC and SDS-PAGE. A similar explanation for the protein A purified MAb solutions, however, seems to be unlikely, because the level of aggregated IgG is comparable to the G and I purified MAb solutions which were clear. Based on these results, it is suggested that tnere are two possible explanations for

the P purified IgG solutions showing light scattering: (i) protein A purified materials contained aggregated IgG (< 0.2μm) that stick to the top of both the HPSEC column and polyacrylamide gel, resulting in the same level of aggregated IgG when compared to the G and I purified MAb solutions; (ii) conformational changes of the IgG molecule may contribute to the enhanced absorbance at wavelength > 320 nm (shape and rigidity may affect light scattering). The latter is most likely because light scattering was not observed if IgG was purified from cell culture supernatant using a single protein A affinity chromatography step (data not shown).

In addition, light scattering was also responsible for the reduced A_{280}/A_{254} ratios as can be calculated using the scatter equation (Rickard et al., 1988). Application of the scatter correction at 280 and 254 nm resulted in A_{280}/A_{254} ratios similar to the ratios obtained by G and I purified MAb solutions. Based on these calculations and DNA/RNA determinations (data not shown), the contribution of nucleic acid absorption at 254 nm appeared to be negligible. This is in agreement with the findings that DNA/RNA is effectively removed by protein A affinity chromatography (Levy et al., 1984 ; Jiskoot et al., 1989).

Recently, it has been demonstrated that acid proteases present in cell culture supernatants can degrade the MAb (Schlaeger et al., 1987; Van Erp et al., 1991c,d). This means that the acid proteases should be removed effectively during the purification process. Monitoring of the proteolytic activity during the methods of purification showed that both sodium sulphate (S) and gel filtration (G) yielded IgG solutions containing an acid proteolytic activity. As shown previously (Van Erp et al., 1991d), the cathepsin D like acid proteases consist of low (40-45 kDa) and high (90-105 kDa) molecular mass forms which are (co-)precipitated by the addition of sodium sulphate. This explains the contamination of the S purified IgG solutions with the acid proteases. The presence of the acid proteolytic activity in IgG solutions after a second gel filtration step can be ascribed to the incomplete separation between the high MW proteases and IgG using the Sephacryl S-300 HR column. Although the relevance of this contamination with acid proteases depends on the type of application the MAb will be used for, it can considerably affect MAb properties upon storage (see Fig. 3).

The effect of each particular purification method on MAb binding activity was determined by measuring the affinity of a MAb for its antigen. Equilibrium binding studies with the chromatographically purified MAb solutions showed K_a values which were not significantly different from those found for the corresponding MAbs in cell culture supernatant. These results suggest that the antigen binding site of the chromatographically purified MAbs is not significantly affected by the method of purification. However, comparison of the heterogeneity indices of the chromatographically purified IgG solutions showed a remarkable difference between ion exchange chromatography (I) and the otherwise purified MAb fractions. The

heterogeneity indices of the I purified materials were in general significantly higher than 1 0 which indicate that the MAbs were heterogeneous with regard to affinity constant The reason for this phenomenon is not clear yet. Although the K_a values of the chromatographically purified IgG solutions were similar, the heterogeneity indices suggest that the binding characteristics of the MAbs purified by ion exchange chromatography were (slightly) affected

Application of all purified IgG solutions in a sandwich immunoassay for hCG showed that a second purification step following sodium sulphate fractionation was essential. Besides the fact that it was not possible to prepare colloidally stable gold sol conjugates using S purified MAb OT-3A, the number of false positive results was reduced to zero using the chromatographically purified materials.

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CHAPTER 7

CHARACTERIZATION OF MONOCLONAL ANTIBODIES PHYSICALLY ADSORBED ONTO POLYSTYRENE LATEX PARTICLES

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submitted for publication

Abstract

The physicochemical and immunochemical properties of monoclonal antibodies (MAbs), adsorbed onto polystyrene latex particles, have been investigated. Both native and pH 2 pretreated MAbs were compared before and after immobilization. It was found that the antigen binding capacity of the immobilized, acidic pretreated MAbs was significantly higher than for the immobilized, native IgG molecules. This enhanced antigen binding capacity appeared to be due to a more oriented adsorption of the monomeric, pH 2 treated IgG fraction. Additionally, experiments using $F(ab')_2$ fragments demonstrated that the Fc part of the MAb molecule is of major importance to obtain the enhanced binding capacity. Binding studies showed that the (apparent) affinity of native and pH 2 pretreated MAbs were similar; the K_a values of the immobilized MAbs were higher than for the MAbs in solution.

Introduction

The immobilization of monoclonal antibodies (MAbs) onto solid phase supports has been extensively used in diagnostic test systems such as ELISA, RIA and latex agglutination assays (Tijssen, 1985; Parson, 1981; Bernard et al., 1986; Kakabakos et al., 1990). One of the most common solid supports in immunoassays is polystyrene and methods for immobilization are predominantly based on physical adsorption.

Physical adsorption of MAbs onto solid supports, however, can lead to partial or complete loss of the antigen binding activity as a result of either a) steric hindrance caused by the attachment of the MAb to the solid support at a region close to its active site or b) conformational changes or c) restriction of the intramolecular flexibility imposed by multi-site attachment to the solid support. Nevertheless an improved binding activity of the physically adsorbed MAbs can be obtained by exposing the MAbs to a low pH environment prior to immobilization. Comparable results have been described for polyclonal antibodies (Ishikawa et al., 1981; Conradie et al., 1983).

Relatively few studies have been published dealing with an explanation for this interesting phenomenon which appeared to affect antigen binding capacity and test performance. Recently, a more detailed explanation has been published (Lin et al., 1989) regarding the influence of adsorption onto silica surfaces for native and "pH modified" polyclonal antibodies.

The main objective of this study was to gain more insight in the properties of physically adsorbed MAbs onto a polystyrene surface. Particular interest was paid to the effect of a pH 2 pretreatment by comparing the physicochemical and immunochemical

properties of both native and pH 2 pretreated MAbs before and after immobilization. For this purpose, colloidal polystyrene latex particles were used because of the rapid reaction of the particle bound MAb with its antigen and easy experimental handling.

Materials and Methods

Materials

The mouse monoclonal antibodies used in this study were directed against human chorionic gonadotrophin (hCG) and of isotype IgG-1. The MAbs with different isoelectric points (OT-9A: 6.4-7.2, 7B; 5.9-6.6, 1C; 6.2-6.9, 4D; 6.5-7.0, 4E; 7.0-7.4) were arbitrarily chosen from a large panel of anti-hCG MAbs and the production in serum-free medium using hollow fibre bioreactors has been described elsewhere (Schönherr et al., 1987). The MAbs were purified by either sodium sulphate precipitation (20%, w/v) followed by a Sephacryl S-200 HR gelfiltration or by protein A affinity chromatography (Van Erp et al., 1991a). Highly purified hCG (12 100 IU/mg) was obtained from Diosynth B.V., Oss, The Netherlands. The (hydrophobic) negatively charged and red coloured polystyrene latex was supplied by AKZO-CR (Arnhem, The Netherlands). The diameter of the latex was 797 nm as determined by quasi elastic light scattering and the latex concentrations were determined using the dry weight method. The affinity purified goat antibodies specific for Fc and $F(ab')_2$ of mouse IgG, and bovine serum albumin (BSA) Boseral R were obtained from Organon Teknika B.V. (Boxtel, The Netherlands). Sephacryl S-200 HR, S-300 HR, Protein A-Sepharose CL-4B and molecular weight standards for high performance size exclusion chromatography (thyroglobulin, 669 000; ferritin, 440 000; IgG, 150 000; BSA, 67 000; ovalbumin, 43 000 and chymotrypsinogen-A, 25 000) were purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical reagent grade quality.

Protein determinations

Protein concentrations were measured by UV-spectroscopy at 280 nm, using the extinction coefficients of 0.39 $(cm \cdot mg/ml)^{-1}$ for highly purified hCG and 1.45 $(cm \cdot mg/ml)^{-1}$ for IgG and $F(ab')_2$ fragments. The extinction coefficient of 1.45 $(cm \cdot mg/ml)^{-1}$ was used also for the pH 2 treated IgG materials since no significant changes occurred in absorbance for native and pH 2 treated MAbs at a concentration of 0.5 mg/ml. The molecular mass of hCG, IgG and $F(ab')_2$ fragments were taken as 38 kDa (Moyle et al., 1983), 150 kDa and 100 kDa, respectively.

pH 2 treatment of IgG

Purified MAb solutions were diluted with 0.05 M glycine/HCl buffer pH 2.0 to a final concentration of 0.5 mg/ml. After incubation of the MAb solution at 0-4°C for 1 h its pH was adjusted to 7.0 by the addition of 0.1 M NaOH.

Adsorption of MAbs onto the latex particles

The MAbs were physically adsorbed onto the polystyrene latex particles by a modification of the method of Fritz and Rivers (1972).

A constant amount of native or pH 2 treated IgG (0.33 mg in 1.6 ml of a 5 mM sodium phosphate buffer pH 7.0 containing 0.1 g/l Thiomersal) was added dropwise and under continuous stirring to a polycarbonate tube containing 1.6 mL latex dispersion in the same phosphate buffer representing a surface area of 0.15 m^2 (1.33% w/w). The samples were incubated during 1 h at 30°C under continuous stirring, and were centrifuged subsequently during 15 min (10^4 N/kg). A sample was taken from the supernatants to determine the MAb concentration, while the sediment was resuspended in the 5 mM sodium phosphate buffer supplemented with 2 g/l BSA and incubated for another 30 min at 30 °C. After a second centrifugation step, the pellet was resuspended in 5 mM phosphate buffer pH 8.0 containing 0.1 g/l Thiomersal and 1 g/l BSA to a latex concentration of 0.85 % (w/w). The concentration of free IgG in the supernatants after adsorption (coating) was determined by sol particle immunoassay (SPIA) for mouse IgG (Leuvering et al., 1983; Van Erp et al., 1991b). The amount of MAb/latex surface (mg/m^2) was calculated using these values and the original MAb concentrations.

Quasi elastic light scattering

Quasi elastic light scattering has been performed using a Coulter Nanosizer (Coulter Electronics, Mijdrecht, The Netherlands) to determine the hydrodynamic diameter of colloidal latex particles. Before measuring, the samples were diluted by a factor of 200 with 5 mM sodium phosphate buffer, pH 8.0.

High performance size exclusion chromatography (HPSEC)

HPSEC was performed on a Zorbax GF-250 column (Dupont Nemours B.V., Den Bosch, The Netherlands) using a 0.2 M NaH_2PO_4/K_2HPO_4 buffer pH 7.0 and UV monitoring at 206 nm as has been described previously (Van Erp et al., 1991b).

Purification of IgG aggregates by gel filtration

The pH of the acidified MAb solutions (pH 2.0) was adjusted to pH 7.0 (total volume: 15 ml). These solutions were applied to a Sephacryl S-300 HR column (bedvolume, 480 ml; K26/100 column, Pharmacia) which was equilibrated in 0.05 M sodium phosphate buffer pH 7.0 containing 0.15 M NaCl and 0.1 g/l sodium azide. The flow rate used was 0.8 ml/min and the effluent was monitored at 280 nm. The fractions (3 ml) containing aggregates of IgG were pooled and concentrated using Centricon 30 microconcentrators (Amicon, Danvers, MA, USA). Subsequently, a buffer change of the IgG solutions was performed on a PD 10 column (Pharmacia, Uppsala, Sweden) with 5 mM sodium phosphate buffer pH 7.0 containing 0.1 g/l Thiomersal. The total IgG recovery was >90% as calculated from the absorbance at 280 nm.

Preparation of $F(ab')_2$ fragments

The $F(ab')_2$ fragments of IgG were prepared as described elsewhere (Van Erp et al., 1991a).

Coating of microtitration plates

Goat anti-mouse (GaM) IgG was dissolved in the "coat buffer" (5 mM NaH_2PO_4, 0.14 M NaCl, 10 g/l PEG 8 000 and 60 g/l Saccharose, pH 7.4), at a concentration of 5.4 μg/ml. 135 μl of this solution was pipetted into each well of a microtitration plate (Greiner, Alphen a/d Rijn, The Netherlands) and incubated overnight at 4°C. After aspiration, the wells were incubated with coat buffer (300 μl/well) supplemented with 0.1% (w/v) BSA during 2 h at room temperature. Subsequently, the wells were washed two times with coat buffer. By this procedure I: goat anti-(mouse IgG/Fc), and II: goat anti-(mouse IgG/$F(ab')_2$) coated microtitration plates were prepared.

Indirect determination of IgG orientation

A dilution series of a MAb-latex conjugate (0.85% w/w) was made in 0.05 M glycine buffer pH 8.5. Samples (100 μl) of the dilution series were pipetted in four fold into the wells of type I and II coated microtitration plates. The plates were sealed and incubated during 2 h at 37°C. Subsequently, the wells were aspirated followed by four washes with 300 μl PBS-Tween (20 mM phosphate buffer pH 7.2 containing 0.13 M NaCl and 0.05% (v/v) Tween-20). Finally, 110 μl of 0.1 M NaOH was added to the wells in order to

dissociate the immune complexes and redisperse the bound anti-hCG latex conjugate. After 15 min the absorbance of each well was measured at 540 nm. Wells incubated with a BSA-latex conjugate were taken as blank.

ELISA for mouse IgG

Dilution series of both native and pH 2 treated MAb solutions were prepared in PBS buffer. Samples (100 μl) were pipetted in four fold into the wells of type I and type II coated microtitration plates and incubated at RT (18-24°C) for 30 min. The microtitration plates were then washed three times with PBS-Tween (300 μl/well) and incubated at RT during 30 min with 100 μl/well sheep anti-mouse (SaM) IgG hRP conjugate, 1:3 000 diluted in PBS-Tween. The excess of enzyme labelled antibody was removed by another three washes with PBS-Tween and subsequently 100μl of the substrate solution (0.45 mM 3,3',5,5'-tetramethylbenzidine, 1.5 mM H_2O_2 and 0.1 M sodium acetate pH 5.5) was added. After colour development (30 min), the reaction was terminated by adding 100 μl of 2 M H_2SO_4. The absorbance of each well was measured by 450 nm.

Determination of antibody affinity

The affinity constants (K_a) of the MAbs in solution were determined as previously described (Van Erp et al., 1991c). After incubation of a constant amount of MAb (4.5 nM, both native and pH 2 treated IgG) with various concentrations of hCG, the free and MAb bound hCG were separated with the aid of an excess of rabbit anti-mouse IgG coupled to cellulose particles (DASP; double antibody solid phase). The free hCG was subsequently determined using a SPIA agglutination procedure for hCG.

The determination of the (apparent) K_a values of the immobilized MAbs was essentially similar. After two washes of the latex conjugates with assay buffer, a constant surface area of 2.25 cm^2 was incubated with various concentrations of hCG during 4 h at RT. Bound and free hCG were subsequently separated by centrifugation during 10 min at 10^5 N/kg. The free hCG concentration in the supernatant was determined using the SPIA agglutination procedure for hCG. Experiments with both normal mouse IgG and BSA coated latices showed that non-specific adsorption of hCG onto the latex conjugates was negligible for the assay conditions used. All experiments were performed at least in duplicate.

Calculation of binding parameters

The binding of antigen (hCG) to immobilized MAb does not necessarily reflect the corresponding interaction between Ag and MAb in solution (Stenberg and Nygren, 1988). The assumptions have been made that the MAb/Ag interaction at a solid-liquid interface is governed by the Law of Mass Action (no mass transport limitations) and reaches equilibrium. In this study, the binding of antigen to immobilized MAbs is quantitatively expressed by an apparent affinity constant.

The (apparent) K_a values for anti-hCG MAbs and hCG were obtained by using the Scatchard and Sips equations (Steward, 1986; Van Erp et al.,1991c). For homogeneous aqueous solutions, r is defined as mol Ag/mol MAb. In case of heterogeneous systems (latex bound MAb) r is calculated as mol Ag bound per mol of effective MAb. The effective number of MAb is taken to be equivalent to the maximum amount of bound Ag, since hCG is a monovalent Ag for each particular MAb used.

Results

Monitoring of the pH 2 pretreatment of different MAbs

HPSEC analyses

As shown in Fig. 1, pH 2 treatment of anti-hCG MAbs yielded a considerable amount of aggregates immediately after adjustment of the pH to 7.0. The major part of these aggregates consisted of di- and trimeric IgG. The level of aggregation, however, was different for each particular MAb and varied between 20-42% of total peak area.

In addition, storage of the treated MAbs at 4-8°C during 24 h showed no decrease in the extent of aggregation indicating that the aggregation is not reversible upon prolonged incubation in solution (pH 7.0) at 4-8°C (data not shown).

Affinity determinations

The effect of pH 2 treatment on the MAb affinity in solution was determined by comparing the affinity constants (K_a) of native and pH 2 treated MAbs. The results are presented in Table I and show that the acid pretreated MAbs retained their affinity in comparison with the native MAbs. Furthermore, comparison of the average number of binding sites per MAb molecule in solution (hCG/MAb ratio) revealed that no or only a slight decrease in active binding sites occurred after pH 2 treatment.

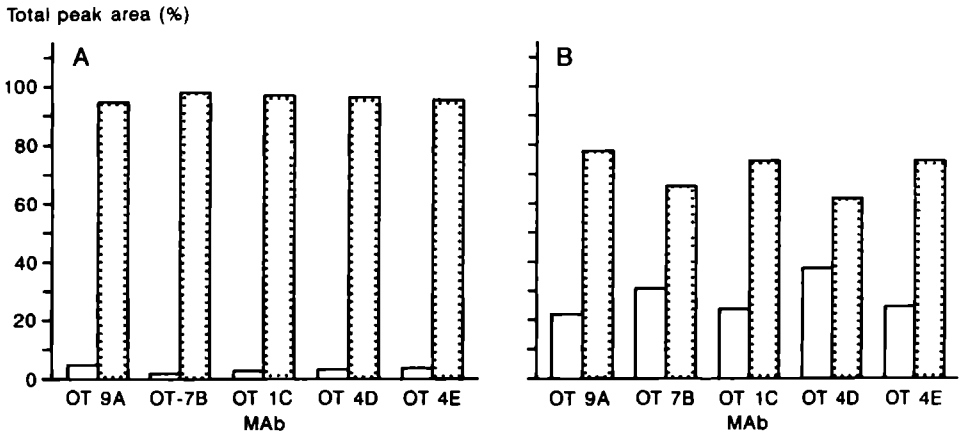

Fig 1 Monitoring of the pH 2 treatment of monoclonal antibodies by HPSEC A) Purified MAb solutions B) pH 2 treated MAb solutions followed by adjustment to pH 7 0 and immediate analysis The content of monomeric (shaded bars) and aggregated IgG (open bars) are expressed as the percentage of total peak area (corrected for salt peaks)

Table I

The effect of pH 2 treatment on MAb affinity in solution

MAb	pH 2 treatment[a]	Scatchard[b] K_a (l/nmol)	Sips[b,c] K_a (l/nmol)	Ratio[d] hCG/MAb
OT-9A	-	1 8	1 8	2 0
	+	1 8	1 9	1 9
OT-7B	-	1 9	2 0	2 1
	+	2 0	2 0	1 9
OT-1C	-	1 6	1 5	2 1
	+	1 4	1 6	1 9
OT-4D	-	10 7	10 7	2 2
	+	10 7	10 5	2 0
OT-4E	-	5 2	5 3	1 9
	+	5 4	5 5	1 9

a Native (-) and pH 2 treated (+) MAb
b The values are the means of duplicates
c The heterogeneity Indices obtained from the Sips equation were not significantly different from 1
d Mol Ag bound per mol of MAb

Affinity

The main advantage of a latex particle bound antibody is that the mobile particle will allow a rapid reaction with the antigen and it was found that an incubation time of 3 h was sufficient for the system to reach (at least an apparent) equilibrium. Furthermore, the latex conjugates remained colloidally stable in the assay buffer during the 3 h incubation as measured by quasi elastic light scattering.

The measured apparent K_a values of four different, immobilized anti-hCG MAbs are summarized in Table IIA . As can be seen, the (apparent) K_a values of native and pH 2 pretreated MAbs were found to be similar after physical adsorption onto polystyrene latex particles. This indicates that no changes in apparent K_a values occurred after pH 2 pretreatment in comparison with the native MAbs. It must be noted, however, that the (apparent) K_a values of the antigen binding to physically adsorbed MAbs (heterogeneous reaction, Table IIA) were higher than those of the homogeneous reaction in solution (Table I) by a factor which varied slightly between the MAbs.

Antigen binding capacity

No preferential adsorption of either native or pH 2 pretreated MAb solutions was observed (Table IIA). Nevertheless, the antigen binding capacities were considerably different (Fig. 2). As summarized in Table IIA, the highest binding capacities as well as maximum hCG/Mab ratios were obtained for the pH 2 pretreated MAb fractions.

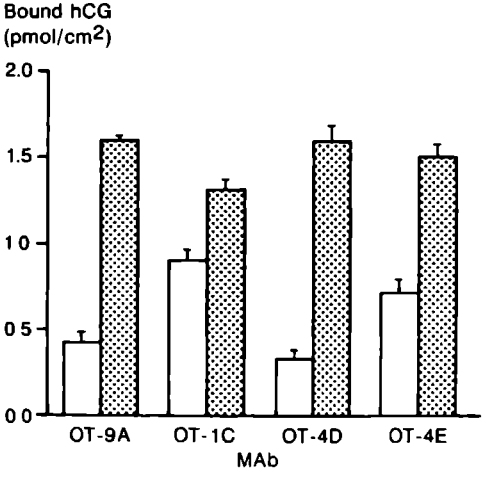

Fig. 2. The hCG binding capacities of native (open bars) and pH 2 treated (shaded bars) MAb latex conjugates. The data represent mean + standard deviation (error bars) of three repeated experiments.

Contribution of the IgG aggregates to the enhanced antigen binding capacity

As described above, the pH 2 treatment yields aggregation of the MAbs. These aggregates of IgG, however, may result in a better accessibility of the MAb binding sites by a decrease in steric hindrance (spacer effect). In order to determine whether the IgG aggregates and/or the monomeric IgG, both obtained after pH 2 treatment, are responsible for the enhanced antigen binding capacity, the following adsorption experiments were performed with purified (Sephacryl S-300 HR gel filtration) aggregates and monomeric pH 2 treated IgG.

Equal amounts (0.33 mg, based on A_{280} nm) of both purified monomeric IgG and aggregates were added to the polystyrene latex surface (0.15 m^2). The amount of adsorbed "IgG" was calculated from the SPIA agglutination procedure for mIgG. The results are summarized in Table IIB. For all MAbs tested, the amount of adsorbed monomeric IgG was slightly lower in comparison with the IgG aggregates. In spite of this lower IgG concentration per latex surface area, the monomeric pH 2 treated IgG latex conjugates had a much higher antigen binding capacity than the aggregates. These results suggest that the aggregates of IgG obtained after the acidic pretreatment do not contribute (significantly) to the enhanced antigen binding capacity of the latex conjugates by formation of a so-called spacer.

Immobilization of $F(ab')_2$ fragments

In order to examine the effect of MAb structural integrity on the enhanced antigen binding capacity, native and pH 2 treated $F(ab')_2$ fragments of three anti-hCG MAbs were adsorbed to the polystyrene latex particles. The amount of adsorbed fragments was determined by the SPIA agglutination procedure for mouse IgG using native and pH 2 treated $F(ab')_2$ fragments for the respective standard curves. Table IIC shows that equal amounts of both native and pH 2 treated fragments adsorbed to the latex surface although less than found for the intact MAbs (Table IIA).

Moreover, it is remarkable that no significant differences in apparent K_a values and Ag binding capacities were observed between native and pH 2 treated $F(ab')_2$ fragments. This suggests that the Fc region is essential to obtain an enhanced antigen binding capacity after pH 2 treatment, when the antibody is adsorbed to latex.

MAb orientation on the latex surface

The increased antigen binding capacity after pH 2 pretreatment might be due to a better orientation of the IgG molecules on the latex surface (The Fab segments more

Table II

Summary of surface concentrations, K_a values and binding capacities of physically adsorbed native and pH 2 treated MAbs (A and B) and fragments (C) on polystyrene latex particles

Table No.	MAbs	pH 2 treat-ment[a]	Surface conc. ($\mu g/cm^2$)	K_a values[b] (l/nmol)	Binding capacity (pmol/cm^2)	Ratio[c] hCG/MAb
A	OT-9A	-	20	4.3	0.4	0.3
		+	20	4.6	1.6	1.2
	OT-1C	-	20	3.4	0.9	0.7
		+	21	3.1	1.3	1.0
	OT-4D	-	21	26	0.3	0.2
		+	21	30	1.6	1.2
	OT-4E	-	20	12.4	0.7	0.5
		+	21	13.4	1.5	1.1
B	OT-9A	A	21	4.5	0.5	0.4
		M	19	4.4	1.1	0.9
	OT-1C	A	21	3.6	0.9	0.6
		M	20	3.3	1.2	1.0
	OT-4D	A	20	28	0.4	0.3
		M	19	25	1.1	0.9
	OT-4E	A	20	13.1	0.3	0.2
		M	19	14.3	1.3	1.0
C	OT-9A	-	8	4.3	0.1	0.1
$F(ab')_2$		+	8	4.9	0.1	0.1
	OT-1C	-	12	3.3	0.4	0.3
		+	13	3.6	0.4	0.3
	OT-4D	-	11	26	0.5	0.4
		+	11	33	0.5	0.4

[a] Native MAb (-); pH 2 treated MAb (+) total fraction; aggregated (A) and monomeric (M) MAb fraction after pH 2 treatment.
[b] The (apparent) K_a values are the means of duplicates (Scatchard equation).
[c] The highest amount of bound Ag (mol) per total amount of immobilized MAb (mol).

oriented towards the solvent) Therefore, the orientation of the MAbs after adsorption to latex was investigated by incubation of the latex conjugates in microtitration plates previously coated with goat antibodies specific for either Fc or $F(ab')_2$ of mouse IgG The results are shown in Fig 3 It was found that the anti-Fc coated (type I) microtitration plates bound more latex particles when coated with pH 2 pretreated MAb rather than native MAb, whereas the opposite was obtained for the anti-$F(ab')_2$ coated (type II) plates Additionally, no preferential binding of either native or acidic pretreated MAb to the coated microtitration plates (type I and II) was observed by experiments using MAbs in solution (both native and pH 2 treated) and subsequently SaM IgG HRP conjugate (Fig 4). Similar results were obtained with SaM IgG coated microtitration plates indicating that there was also no preferential binding of SaM IgG to native or pH 2 treated MAbs (data not shown) These results suggest that there are more Fab segments oriented towards the solvent when pH 2 treated MAbs are adsorbed to latex

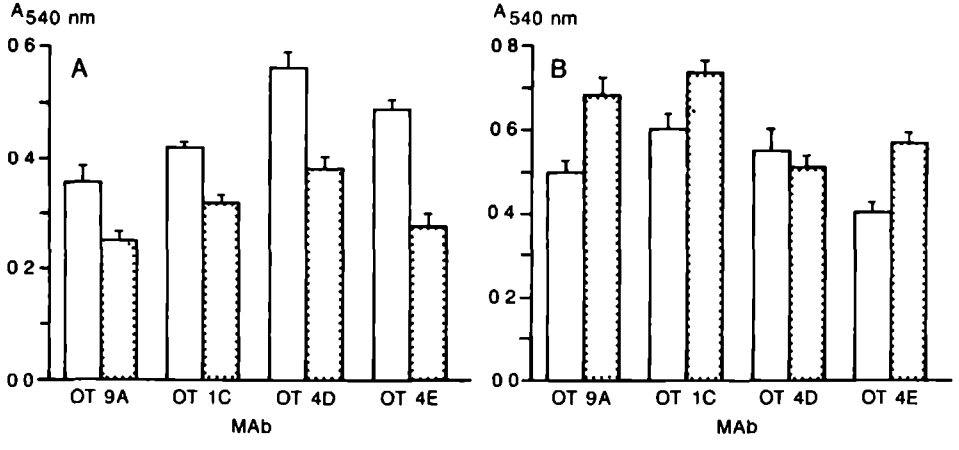

Fig 3 Binding of both native (open bars) and pH 2 treated (shaded bars) MAb latex conjugates (1 5 dilution) to microtitration plates previously coated with GaM Fc specific (A) and $F(ab')_2$ specific (B) IgG fractions The values are corrected for the blank (BSA coated latex conjugate) and represent mean + standard deviation (bars) of quadruplicate determinations

Discussion

The pH 2 treatment of MAbs prior to adsorption onto the polystyrene latex surface resulted in an increased antigen binding capacity of the final conjugate as compared to the native MAbs based conjugate Four possible explanations for the increased binding

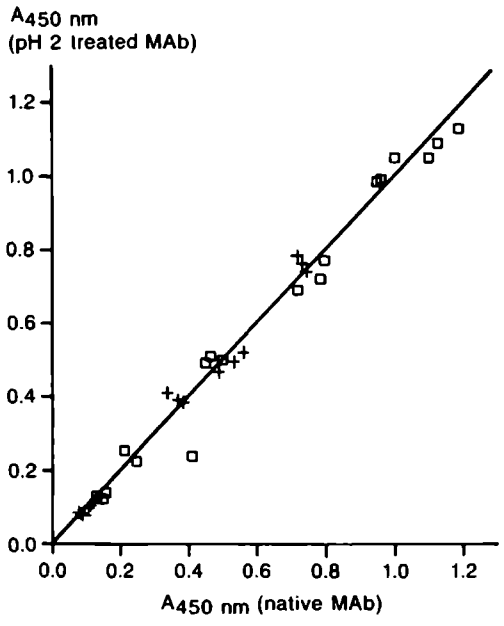

Fig. 4. Binding of native and pH2 treated MAb (OT-4E) to type I (+) and type II (□) coated microtitration plates. The correlation coëfficient (r) for both type I and type II coated microtitration plates is 0.99. The line represents Y=X.

capacity after pH 2 treatment are: (1) an increase in antibody affinity due to conformational changes in the Ag binding site, (2) an increased solid phase adsorption of pH 2 treated MAbs, (3) the aggregates of MAbs obtained increase the number of accessible binding sites by a spacer effect, (4) changes in structural integrity of the MAb facilitate a more oriented adsorption of the MAb molecule. The validity of each of these explanations are discussed successively.

Binding studies of several anti-hCG MAbs, both in solution and physically adsorbed onto latex particles, demonstrated that no changes in K_a values occurred after a pH 2 pretreatment in comparison with the native MAbs. This indicates that the first explanation is unlikely. The (apparent) K_a values of the immobilized MAbs, however, were higher than of those in solution. The reason for this phenomenon is not yet clear but it is unlikely that the enhancement in K_a values is due to experimental differences since the same procedure for determining K_a was used for both the MAbs in solution and Mabs adsorbed onto polystyrene latex. On the other hand, the solid phase adsorption might give a favourable conformational change of the MAbs yielding increased K_a values. Another factor could be the increase in the local MAb concentration due to the

immobilization; this effect is absent in the case of MAbs in solution (homogeneous system). Both options are currently investigated.

In contrast with the considerable loss of active binding sites of polyclonal antibodies in solution after low pH treatment (Lin et al., 1989), no or only a slight decrease in total active binding sites was observed for the anti-hCG MAbs in solution. The acidic treatment of a MAb prior to its physical adsorption gives a positive contribution to the total number of active binding sites per surface area (Ag binding capacity).

Conradie et al. (1983) observed an increased solid phase adsorption of polyclonal antibodies after low pH treatment. In the present experiments, however, equal amounts of both native and pH 2 treated MAbs were adsorbed to the polystyrene latex particles indicating that the second explanation is less likely for the enhanced antigen binding capacities observed in this study. The occurrence of a higher desorption (leakage) of the native immobilized MAb as compared to the pH 2 treated MAb is also unlikely as determined by the SPIA agglutination procedure for mouse IgG.

Monitoring of the pH 2 treatment by HPSEC showed that exposure to a low pH environment followed by adjustment to pH 7.0 caused MAb aggregation. Application of aggregated MAbs could increase the number of accessible binding sites by a spacer effect (decrease of steric hindrance by the solid phase). However, experiments with either aggregated or (pH modified) monomeric IgG fractions, both adsorbed onto the latex particles, demonstrated that the highest number of active binding sites were found for the monomeric pH 2 treated IgG latex conjugates. The IgG aggregates obtained after pH 2 treatment obviously did not significantly enhance the antigen binding capacity of the physically adsorbed MAbs. Explanation 3 can therefore be ruled out.

Infrared- (Abaturov et al.,1969), circular dichroism- (Conradie et al., 1983) and fluorescence spectroscopy studies (Lin et al., 1989), have shown that IgG molecules in solution undergo structural changes upon lowering the pH. These changes mainly occur in the Fc part as indicated by infrared and circular dichroism measurements using Fab and Fc fragments. The lower conformational stability of the Fc fragment compared to that of the Fab fragments is confirmed by both the data on proteolytic hydrolysis (Tijssen, 1985) and the similar K_a values for native and pH 2 treated MAbs as described in this paper. Also the data obtained from physically adsorbed $F(ab')_2$ fragments clearly demonstrate the involvement of the Fc region in the increased Ag binding capacity upon pH 2 treatment of the MAbs prior to immobilization (Table IIC).

Finally, the indirect information with respect to the orientation of the physically adsorbed MAbs showed that the Fc region is more oriented towards the solvent for the native MAb coated latex particles, whereas the Fab segments are more oriented towards the liquid phase for the pH 2 pretreated MAbs.

In conclusion, all described observations support explanation 4, namely that the pH 2 treatment of a MAb partially modifies the Fc part of the MAb molecule, thereby increasing the local hydrophobicity of the IgG molecule (Vandenbranden et al., 1981) and consequently facilitating the oriented adsorption of the modified monomeric IgG fraction such that the Fab segments are more oriented towards the solvent, favouring antigen binding capacity

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CHAPTER 8

SUMMARY/SAMENVATTING

Summary

The development of the hybridoma technology by Köhler and Milstein in 1975 has provided a method of generating virtually unlimited quantities of a single monoclonal antibody (MAb) of predefined specificity, affinity and isotype. These monoclonal antibodies which possess unique advantages over polyclonal antisera, have rapidly gained recognition as powerful tools for applications in both biomedical and non-biomedical fields. One of the major applications of monoclonal antibodies are the *in vitro* immunodiagnostic kits for the measurement and determination of drugs, hormones and infectious disease markers. They serve as diagnostic assays that take full advantage of the particular monoclonal antibody properties such as specificity, quantity, sensitivity and ability to identify different epitopes. However, while diagnostic systems based on the use of MAbs were being developed, it has become increasingly apparent that monoclonal antibodies are not eternally consistent. Consequently, even when using MAbs, reproducibility is not always ensured.

As indicated in Chapter 1 (which outlines the background of this study and reviews the relevant literature), monoclonal antibodies are complex molecules which can undergo a variety of modification and degradation processes during production, purification, storage and immobilization onto solid supports. In order to control test development and production it is important to gain insight in the significance of these modifications in terms of physicochemical and immunochemical properties. Since antibody affinity (the strenght of antibody/antigen interaction) is of major importance in the performance of immunoassays, special attention has been paid to the occurrence of changes in MAb affinity.

The objectives of the investigations described in this thesis were: (1) the development of a procedure to determine the affinity constants of MAbs for high MW antigens both native and in solution and, (2) to monitor the properties of MAbs during (large scale) production, purification and application in diagnostic test systems. This involves determining which parameters need to be monitored and when to control adequately MAb production and test development.

In Chapter 2 the application of a sol particle immunoassay to the determination of affinity constants of monoclonal antibodies is described. In this procedure, a fixed concentration of MAb is incubated in solution with a concentration dilution series of the antigen until equilibrium is reached. The antibody-antigen complexes in the equilibrium mixtures are separated from free Ag by adding an excess of rabbit anti-mouse IgG coupled to cellulose particles (DASP) followed by centrifugation. Subsequently, the free antigen concentration in the supernatant is measured using a gold sol particle agglutination assay (SPIA) in a microtitration plate format.

The present method permits the determination of antibody affinities in the range of 10^6-10^{10} M^{-1} and the values of K_a determined by this procedure for anti-hCG/hCG interactions were found to be similar to the values obtained by a competitive RIA procedure. The method described, has a number of advantages as compared to the commonly used techniques to measure Ab affinity. First, the initial reaction is performed in solution and no labelling of either antibody or antigen is required. This means that no modification of antibody affinity due to immobilization or labelling occurs. Furthermore, the procedure is easy to perform, applicable to high molecular weight antigens and permits screening of cell culture supernatants (see also Chapter 4) and a variety of conditions such as buffer composition and temperature.

Equilibrium binding studies demonstrated that some MAbs specific for hCG can bind in a "positive cooperative" way (convex Scatchard plots). This phenomenon of binding enhancement, which could not be ascribed to artefacts in the procedure, has been further investigated.

In Chapter 3 some aspects are described to support a role for the Fc and hinge region in the cooperative binding behaviour of (some) MAbs directed against hCG. It is demonstrated that the apparent positive cooperativity (convex Scatchard plots) was strongly dependent upon concentration and temperature. This dependence of convexity upon concentration is a characteristic phenomenon for associating systems. Since addition of normal mouse IgG did not increase the convexity of the almost linear Scatchard plot, a concentration dependent association between anti-hCG MAbs, mediated or facilitated by prior antigen binding, is hypothesized. This hypothesis was confirmed by HPSEC analysis of the equilibrated hCG/anti-hCG mixtures. The molecular mass of the MAb/Ag complex changed from 226 kDa to 450 kDa upon increasing the hCG/anti-hCG ratio. Moreover, the apparent positive cooperativity was strongly reduced using $F(ab')_2$ fragments of IgG and became undetectable when the MAb was replaced by the corresponding Fab fragment.

All of the present observations are in agreement with the proposed hypothesis of a non-covalent Fc-Fc interaction between anti-hCG MAbs, mediated or facilitated by prior Ag binding. This cooperative interaction between one particular monoclonal antibody and hCG, however, is to be distinguished from the previously described mechanisms for cooperative binding of two MAbs having distinct specificities for hCG.

Chapter 4 is concerned with the monitoring of the MAb production during long-term cultivation of hybridoma cells under serum-free conditions using hollow fibre bioreactors. Several analytical techniques were used to collect information on quantitative and qualitative aspects of the produced MAbs, cell viability, cell lysis and proteolytic activity.

During the culture periods of the hybridoma cells producing MAb OT-1C and 3A, the concentration of MAb showed a decreasing trend with a concomitant increase of IgG fragments (A, 43 kDa; and B, 23kDa). This effect, however, was much larger for MAb OT-1C than for OT-3A. The major IgG fragments, of which fragment A could be reduced by mercaptoethanol, did not bind the antigen (hCG) and the molecular masses were significantly different from the particular MAb light and heavy chains. Additionally, the results clearly show that cell lysis, as measured by LDH appearance, correlates with the appearance of (an acid) proteolytic activity and IgG fragments (A and B). This suggests that the MAbs undergo (some) degradation as a result of proteolytic enzyme(s) released from dead or lysed cells.

The physicochemical and immunochemical properties of the 'intact' MAbs (such as molecular mass, IEF patterns and affinity) did not change significantly over the course of the cultures.

Chapter 5 describes the identification and purification of the proteolytic enzyme(s) found in cell culture supernatants from long-term cultivation of hybridoma cells in hollow fibre bioreactors. Several methods were used to characterize the proteolytic activity and the main results were: (1) the acid proteolytic activity showed a maximum around pH 3 and declined essentially to zero at pH 8.0; (2) the activity was specifically inhibited by pepstatin A; (3) the acid proteases consisted of two sets of closely spaced bands with apparent molecular masses of 40-45 kDa and 90-105 kDa respectively; (4) the low and high molecular mass protease bands were reactive with anti-human cathepsin D; (5) the IEP values of the acid proteases ranged from 4.6-6.5. All presented observations indicated that the acid protease(s) were similar to lysosomal cathepsin D. Purification of the acid protease(s) from hybridoma cells and subsequent incubation with monomeric MAb solutions at pH 5 yielded IgG fragments similar to those found in serum-free hollow fibre cell culture supernatants. These results indicated that the IgG fragments mentioned in chapter 4 are the result of degradation by cathepsin D like protease(s) released after cell death or cell lysis. The presence of these protease(s) may strongly influence the quantity, quality and batch to batch consistency of the MAb products. Therefore, the protease(s) should be specifically inhibited during the culture period or removed/inhibited immediately after harvesting.

Chapter 6 deals with the effect of methods of purification on both MAb properties and purity. Three anti-hCG MAbs, two batches of each, were purified from serum-free hollow fibre cell culture supernatants. Starting with sodium sulphate fractionation as a pre-purification step, final purification was performed using Sephacryl S-300 HR gel filtration, Mono Q ion exchange chromatography or protein A affinity chromatography. The impact of these methods of purification on the physico-/immunochemical properties

of the MAb products was investigated by a combination of techniques including HPSEC, SDS-PAGE, IEF, UV/VIS spectrophotometry, affinity measurements, protease assay, DNA assay and performance in immunoassays.

In spite of purification conditions ranging from mild to rather drastic, no significant effect on the (mean) K_a values was found when compared to the K_a values of the particular MAbs in cell culture supernatant. However, the heterogeneity indices for ion exchange purified MAbs were in general significantly higher than 1.0, indicating that the MAbs were heterogeneous with regard to affinity constant. The purity of the IgG solutions after sodium sulphate precipitation ranged from 86-90% and was more than 95% for the chromatographically purified solutions as determined by HPSEC and SDS-PAGE. Protein A affinity chromatography, however, yielded MAb solutions showing light scattering, responsible for a reduced A_{280}/A_{254} ratio. In addition, IgG fractions free of proteolytic activity were obtained only when ion exchange or protein A affinity chromatography was used as a polishing step. Finally, application of chromatographically purified IgG in a sandwich immunoassay for hCG reduced the number of false positive results as compared to the assays based on sodium sulphate purified IgG solutions.

Immobilization of monoclonal antibodies onto solid phase materials has been widely used in diagnostic test systems such as RIA, ELISA and latex agglutination assays. In chapter 7, the physicochemical and immunochemical properties of MAbs, physically adsorbed onto polystyrene latex particles are descibed. Both native and pH 2 pretreated MAbs were compared before and after immobilization. It was found that the antigen binding capacity of the physically adsorbed, pH 2 pretreated MAbs was substantially higher than for the adsorbed, native MAb molecules. This enhanced antigen binding capacity appeared to be due to a more oriented adsorption of the monomeric, pH 2 pretreated IgG fraction. In addition, experiments using $F(ab')_2$ of the MAbs clearly showed that the Fc region of the MAb molecule is of major importance to obtain the enhanced binding capacity.

Binding studies demonstrated that the (apparent) affinity constants of native and pH 2 pretreated MAbs were similar, suggesting that the original conformation of the Ag binding sites is preserved. However, the K_a values of the immobilized MAbs were higher than for the MAbs in solution. All observations described suggest that the pH 2 treatment of a MAb partially modifies the Fc part of the MAb molecule, thereby increasing the local hydrophobicity of the IgG molecule and consequently facilitating the oriented adsorption of the modified monomeric IgG fraction such that the Fab segments are more oriented towards the solvent, favouring antigen binding capacity.

Sinds de ontwikkeling van de hybridoma technologie in 1975 door Köhler en Milstein, waardoor het mogelijk werd monoklonale antistoffen met een unieke specificiteit in grote hoeveelheden te produceren, is het gebruik van monoklonale antistoffen in zowel de biomedische als niet biomedische gebieden enorm toegenomen. Een van de belangrijke toepassingsgebieden van monoklonale antistoffen zijn de *in vitro* immunodiagnostische testen voor het bepalen van onder andere hormonen en infectieuze ziekten. Deze diagnostische testen maken volledig gebruik van de bijzondere voordelen van monoklonale antistoffen zoals specificiteit, affiniteit en hoeveelheid. Ondanks de grote voordelen van monoklonale antistoffen ten opzichte van de polyklonale antisera, blijkt dat (ook) monoklonale antistoffen zich soms onvoorspelbaar gedragen bij het ontwikkelen van immunodiagnostica.

Zoals beschreven in de introductie (Hoofdstuk 1) van dit proefschrift zijn monoklonale antistoffen complexe eiwitmoleculen die een verscheidenheid aan modificatie en degradatie processen kunnen ondergaan tijdens productie, zuivering, opslag, en immobilisatie aan een oppervlak. Om uiteindelijk de ontwikkeling en produktie van immunodiagnostica beter te kunnen controleren is het belangrijk om meer inzicht te verkrijgen in de mate waarin de modificaties met betrekking tot de fysische- en immunochemische eigenschappen van monoklonale antistoffen voorkomen. Speciale aandacht gaat hierbij uit naar veranderingen in de affiniteit (een maat voor de sterkte van de antistof-antigen interactie) van een monoklonale antistof, omdat deze van groot belang is voor de gevoeligheid en specificiteit van een immunodiagnostische test.

De doelstellingen van het in dit proefschrift beschreven onderzoek waren: (1) Het ontwikkelen van een snelle methode om in oplossing (zowel buffer als celkweek supernatant) de affiniteitsconstante te bepalen voor de interactie tussen natieve monoklonale antistoffen en hoog moleculaire antigenen. (2) Het vervolgen (monitoren) van monoklonale antistof karakteristieken tijdens produktie (op grote schaal), zuivering en toepassing in diagnostische testsystemen. Hierbij gaat het om het bepalen welke parameter(s) en wanneer, gemeten moet(en) worden om de ontwikkeling en productie van immunodiagnostische testen beter te kunnen controleren.

In hoofdstuk 2 wordt een eenvoudige en snelle methode beschreven om de affiniteitsconstante te bepalen voor de interactie tussen een monoklonale antistof en een hoog moleculair antigeen (hCG) onder evenwichtsomstandigheden. Hiertoe werd een constante hoeveelheid monoklonale antistof (anti-hCG) in oplossing geincubeerd met een verdunningsreeks van antigeen (hCG). Na instelling van het evenwicht werd het vrije antigeen gescheiden van het gebonden antigeen door een overmaat aan konijn anti-

muis IgG, gekoppeld aan cellulosedeeltjes (DASP), toe te voegen. Na centrifugatie werd het vrije antigeen (hCG) in het supernatant gemeten met behulp van de SPIA agglutinatie procedure. De beschreven procedure heeft een aantal voordelen in vergelijking met andere methoden om affiniteitsconstanten van monoklonale antistoffen te bepalen. Ten eerste vindt de antistof-antigen interactie plaats in oplossing en wordt er geen gebruik gemaakt van labels. Dit betekent dat er geen modificatie van de (monoklonale antistof) affiniteit optreedt als gevolg van het immobiliseren of conjugeren. Verder is de procedure gemakkelijk uitvoerbaar en bruikbaar voor antigenen met een hoog molekuulgewicht. Bovendien is de procedure ook geschikt voor bepalingen in celkweeksupernatanten (zie hoofdstuk 4) en om de invloed van verschillende omstandigheden zoals buffersamenstelling en temperatuur te onderzoeken.

De affiniteitsconstanten voor anti-hCG/hCG interacties bepaald met behulp van deze procedure, waren vergelijkbaar met de waarden verkregen via een conventionele RIA procedure. Uit de bindingsexperimenten bleek echter dat (enkele) anti-hCG monoklonale antistoffen een (schijnbare) positieve coöperatieve interactie vertoonden met hCG. Deze coöperatieve interactie, die niet te wijten was aan artefacten in de procedure, werd verder onderzocht en de resultaten staan beschreven in het volgende hoofdstuk.

In hoofdstuk 3 worden enkele aspecten beschreven met betrekking tot de coöperatieve bindingskarakteristieken van (enkele) monoklonale antistoffen gericht tegen hCG. De coöperatieve interacties (convexe Scatchard plots) zoals beschreven in hoofdstuk 2 bleken afhankelijk te zijn van concentratie en temperatuur. De concentratie afhankelijkheid van coöperatieve interacties is een karakteristiek fenomeen voor associerende systemen. Omdat er uiteindelijk geen toename van coöperativiteit werd waargenomen na toevoeging van niet-specifiek muize IgG, werd verondersteld dat er een concentratie afhankelijke associatie tussen anti-hCG monoklonale antistoffen plaatsvond, die vergemakkelijkt dan wel gestuurd werd door binding met hCG. Deze hypothese werd bevestigd door de antistof-antigeen reactiemengsels te analyseren met behulp van de HPLC. Het bleek dat de molekuulmassa van het antistof-antigen complex veranderde van 226 kDa naar 450 kDa met toenemende hCG/anti-hCG ratio's. Bovendien was de coöperatieve interactie aanzienlijk minder voor de $F(ab')_2$ fragmenten van de desbetreffende monoklonale antistoffen en verdween zelfs in het geheel na gebruik van Fab fragmenten. Deze gezamelijke resultaten wijzen op een niet covalente Fc-Fc interactie tussen anti-hCG monoklonale antistoffen hetgeen vergemakkelijkt of gestuurd wordt door binding met het antigeen hCG.

Hoofdstuk 4 beschrijft het vervolgen van de monoklonale antistof produktie onder serum-vrije condities in holle vezel bioreactoren. Verschillende technieken werden gebruikt om informatie te verschaffen over de kwalitatieve en kwantitatieve aspecten van de geproduceerde monoklonale antistoffen, levende en dode cellen, cellyse en proteolytische activiteit.

De concentraties aan monoklonale antistoffen daalden naarmate de productie periode langer voortduurde. Deze afname in IgG concentratie ging gepaard met een toename van IgG fragmenten (A; 43 kDa en B; 23 kDa). Dit effect was echter sterker voor monoklonale antistof OT-1C dan voor OT-3A. Deze belangrijkste IgG fragmenten, waarvan fragment A verder gereduceerd kon worden, bleken geen activiteit te bezitten voor hCG en de molekuulmassa's van beide fragmenten waren verschillend van de corresponderende monoklonale antistof lichte en zware ketens. Bovendien werd er een goede correlatie waargenomen tussen cellyse, proteolytische activiteit en het ontstaan van IgG fragmenten. De resultaten wijzen op een degradatie van de monoklonale antistoffen als gevolg van proteolytische enzymen die vrijkomen na afsterven of lyseren van hybridoma cellen.

De fysisch-chemische en immuno-chemische karakteristieken van de "intacte" monoklonale antistoffen (zoals molekuulmassa, isoëlektrische punten en affiniteit) vertoonden daarentegen geen significante veranderingen gedurende de kweekperioden.

Hoofdstuk 5 beschrijft de identificatie en zuivering van de (zure) proteolytische enzymen aanwezig in serum vrije hybridoma celkweek supernatanten van holle vezel bioreactoren. De karakteristieken van de (zure) protease(s) bleken grote overeenkomsten te vertonen met het lysosomale carboxyl protease cathepsine D. Zuivering van de protease(s) uit hybridoma cellen gevolgd door een incubatie met monomeer IgG resulteerde in IgG fragmenten die vergelijkbaar waren met de IgG fragmenten in serum vrije celkweek supernatanten van holle vezel bioreactoren. Deze resultaten geven aan dat de IgG fragmenten zoals beschreven in hoofdstuk 4 het resultaat waren van een proteolytische degradatie door cathepsine D achtige proteases. De aanwezigheid van deze proteases kunnen uiteindelijk een grote invloed hebben op de kwantiteit, kwaliteit en reproduceerbaarheid van het monoklonale antistof product. De proteases moeten derhalve specifiek geremd worden tijdens de productie periode of onmiddelijk verwijderd/geremd worden na het oogsten.

In hoofdstuk 6 wordt het effect van verschillende zuiveringsmethoden op zowel de eigenschappen als zuiverheid van de monoklonale antistoffen beschreven. Drie verschillende tweestaps-zuiveringsprocedures, bestaande uit een natriumsulfaat precipitatie (eerste stap) in combinatie met gelfiltratie, anionenwisselings- of protein A

affiniteits-chromatografie (tweede stap), werden met elkaar vergeleken. Het effect van deze methoden op de fysisch- en immuno-chemische eigenschappen van de monoklonale antistoffen werd onderzocht met spectrofotometrie, SDS-PAGE, isoëlektrische focussering, gelfiltratie, affiniteitsmetingen, protease en DNA bepalingen, en het uiteindelijke "test-gedrag" in een immunoassay.

De zuiverheid van de monoklonale antistof oplossingen na natriumsulfaat precipitatie varieerde tussen 86-90% terwijl de zuiverheid groter was dan 95% voor de chromatografisch gezuiverde monoklonale antistof oplossingen.

Ondanks de verschillende zuiveringscondities, variërend van mild tot drastisch, werd er geen significant verschil waargenomen in de (gemiddelde) affiniteit van een monoklonale antistof. Nochtans waren de bindingskarakteristieken van nagenoeg alle monoklonale antistoffen, gezuiverd via ionenwisselings-chromatografie, verschillend van de monoklonale antistoffen verkregen via de andere technieken. De Sips-plots lieten namelijk heterogeniteits indices zien groter dan één. Dit betekent dat de monoklonale antistoffen gezuiverd via ionenwisselings-chromatografie heterogeen waren met betrekking tot de affiniteitsconstante.

Protein A affiniteits-chromatografie (als tweede stap) resulteerde in monoklonale antistof oplossingen die licht verstrooiden. Deze lichtverstrooing bleek uiteindelijk ook verantwoordelijk voor de verlaagde A_{280}/A_{254} ratio's. Speciale aandacht werd ook besteed aan het verwijderen van de (zure) proteases zoals beschreven in de hoofdstukken 4 en 5. Het bleek dat alleen na ionenwisselings- en protein A affiniteits-chromatografie monoklonale antistof oplossingen werden verkregen die vrij waren van de (zure) proteases. De uiteindelijke toepassing van chromatografisch gezuiverde monoklonale antistoffen in een "sandwich" immunoassay voor hCG reduceerde het aantal fout positieve resultaten in vergelijking met de testen gebaseerd op natrium sulfaat gezuiverde monoklonale antistof oplossingen.

Het immobiliseren van monoklonale antistoffen aan oppervlakken wordt veelvuldig gebruikt in diagnostische systemen zoals RIA, ELISA, SPIA en latex agglutinatie testen. Hoofdstuk 7 handelt over de fysisch-chemische en immuno-chemische eigenschappen van geadsorbeerde monoklonale antistoffen aan polystyreen latex deeltjes. Zowel natief als pH 2 behandelde monoklonale antistoffen werden voor en na immobilisatie vergeleken. Het bleek dat de antigeen-bindingscapaciteit van de geadsorbeerde en pH 2 voorbehandelde monoklonale antistoffen (gericht tegen hCG) aanzienlijk hoger was dan voor de niet behandelde (natieve) monoklonale antistoffen. Deze verhoogde antigeen-bindingscapaciteit bleek toegeschreven te kunnen worden aan een beter georienteerde adsorptie van de pH 2 behandelde monomere IgG fractie. Experimenten met $F(ab')_2$ fragmenten van IgG toonden duidelijk aan dat het Fc gebied van groot belang was bij het verkrijgen van de verhoogde antigeen-bindingscapaciteit.

Bindingsstudies lieten zien dat de (schijnbare) affiniteitsconstanten van natief en pH 2 voorbehandelde monoklonale antistoffen gelijk zijn. Dit betekende dat de oorspronkelijke conformatie van de antigeen-bindingsplaatsen behouden bleef. Desalniettemin waren de (schijnbare) affiniteitsconstanten van de geimmobiliseerde monoklonale antistoffen hoger dan de waarden gemeten voor de monoklonale antistoffen in oplossing. De gezamenlijke resultaten suggereren dat een pH 2 behandeling gedeeltelijk het Fc gebied van een monoklonale antistof modificeert, waarbij de lokale hydrofobiciteit van het IgG molekuul toeneemt met als gevolg dat de geörienteerde adsorptie van pH 2 behandeld monomeer IgG vergemakkelijkt wordt. Dit betekent waarschijnlijk dat de Fab segmenten van het IgG molekuul meer geörienteerd zijn naar de oplossing zodat de antigeen-bindingscapaciteit toeneemt.

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CURRICULUM VITAE

René van Erp werd geboren op 22 maart 1964 te Vught. In 1982 werd het diploma Atheneum-B behaald aan het Maurick College te Vught. In hetzelfde jaar begon hij met de studie Scheikunde aan de Katholieke Universiteit Nijmegen. De doctoraalstudie omvatte de hoofdvakken biochemie (Prof. Dr. H. Bloemendal) en chemische microbiologie (Prof. Dr. Ir. G.D. Vogels). Het doctoraal diploma werd behaald op 14 december 1987. Van december 1987 tot december 1990 was hij werkzaam als assistent-in-opleiding bij aanvankelijk AKZO Pharma te Oss en later bij Organon Teknika B.V. te Boxtel. Gedurende deze periode werd onder leiding van Prof. Dr. H.P.J. Bloemers (Katholieke Universiteit Nijmegen) en Dr. T.C.J. Gribnau (Organon Teknika B.V.) aan het hier beschreven promotieonderzoek gewerkt. Sinds 1 december 1990 is hij werkzaam als project supervisor bij Organon Teknika B.V. te Boxtel binnen de afdeling Screening Systems Research Unit.